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8/27/02
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Robert Michael ROBERTS
Jonathan Andrew GREEN and
Sancai XIE

Serial No.: 09/273,164

Filed: March 19, 1999

For: COMPOSITIONS AND METHODS FOR
EARLY PREGNANCY DIAGNOSIS

Group Art Unit: 1643

Examiner: L. Cook

Atty. Dkt. No.: UVMO:003

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Robert E. Hanson

BRIEF ON APPEAL

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APPENDIX 1: Appealed Claims Prior to Entry of Amendment Under 37 C.F.R. § 1.116

APPENDIX 2: Appealed Claims Following Entry of Amendment Under 37 C.F.R. § 1.116

APPENDIX 3: Exhibits

Exhibit A — Roberts *et al.* (1995)

Exhibit B — Zoli *et al.* (1992)

Exhibit C — Sasser *et al.* (1989)

Exhibit D — Xie *et al.* (1997)

Exhibit E — Gerrie *et al.* (1986)

Exhibit F — Encarta™ online dictionary definition of “about”

Exhibit G — Merriam Webster’s Collegiate Dictionary™ definition of “about”

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BRIEF ON APPEAL

BOX AF

Commissioner of Patents
Washington, D.C. 20231

Sir:

This brief is filed (in triplicate) in response to the Final Office Action mailed on January 2, 2002. The Notice of Appeal was mailed in the case on April 2, 2002. The date for filing the instant Brief is August 16, 2002, based on the receipt of the Notice of Appeal by the Patent and Trademark Office on April 16, 2002 and the Petition for Extension of Time of two-months below. The fees for the brief and the extension of time are enclosed. Please date stamp and return the attached postcard as evidence of receipt.

PETITION FOR EXTENSION OF TIME

Pursuant to 37 C.F.R. §1.136(a), Appellants petition for an extension of time of two months, to and including August 16, 2002, in which to file this Appeal Brief. The fee for the extension is enclosed. Should any other fees be due under 37 C.F.R. §1.17, the Commissioner is

authorized to deduct such fee from or Fulbright & Jaworski Deposit Account No. 50-1212/10012519/10056.

I. REAL PARTIES IN INTEREST

The real parties in interest are the assignee of the case, The Curators of the University of Missouri, and the licensee thereof, Monsanto Company.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-8, 10-14, 30-34 and 182-183 were pending at the time of the final Office Action. Claims 1, 3-9 and 30-34 were finally rejected. However, claim 9 is not pending in the case and thus claim 1, 3-8 and 30-34 are the subject of this appeal. An amendment under 37 C.F.R. §1.116 is being filed concurrently herewith. A copy of the appealed claims prior to entry of the Amendment under 37 C.F.R. § 1.116 is attached as Appendix 1 and a copy of the claims following entry of the Amendment under 37 C.F.R. § 1.116 is attached as Appendix 2.

*conceded claims 9, 15-21, 30-50.
pending claims 35-181 not under consideration.*

IV. STATUS OF AMENDMENTS

No amendments to the claims were entered in the case subsequent to the final Office Action and at the time of filing the instant Brief. Claims 12-14 and 182-183 were amended in Appellants' Amendment and Response to Final Office Action, but the entry of the amendments was refused by the Examiner. An Amendment under 37 C.F.R. § 1.116 is being filed concurrently herewith.

*check - paper #19-L filed 5/29/02
paper #23-D filed 8/1/02*

V. SUMMARY OF THE INVENTION

The invention relates to a method for detecting pregnancy in a bovine animal comprising using an antibody that binds immunologically to at least one pregnancy associated antigen (PAG) that is present in early pregnancy and absent at about two months post-partum. Specification from page 4, line 20 to page 5, line 6. The invention is significant because it allows pregnancy detection early and in animals that have been rebred after calving, which customarily is done within 2 to 3 months post-partum. Specification from page 3, line 26 to page 4, line 16.

VI. ISSUES ON APPEAL

- A. Are claims 1, 3, 5, 6 and 14 anticipated under 35 U.S.C. §102(b) by Roberts *et al.* (1995)?
- B. Are claims 1, 3, 5, 6 and 13 anticipated under 35 U.S.C. §102(b) by Zoli *et al.* (1992)?
- C. Are claims 4, 7 and 8 obvious under 35 U.S.C. §103(a) over Roberts *et al.* (1995) or Zoli *et al.* (1992) in view of Sasser *et al.* (1989)?
- D. Are claims 30-34 obvious under 35 U.S.C. §103(a) over Roberts *et al.* (1995) and Zoli *et al.* (1992) in view of Xie *et al.* (1997) and Gerrie *et al.* (1986)?

VII. GROUPING OF THE CLAIMS

The claims stand or fall together for purposes of the appeal.

VIII. SUMMARY OF THE ARGUMENT

The references cited by the Examiner do not teach all elements of the claims and no teachings have been alleged by the Examiner which could support such a conclusion. Claim 1, upon which each of the appealed claims directly or indirectly depends, describes a method for detecting pregnancy in a bovine animal that comprises use of an antibody that binds immunologically to at least one pregnancy associated antigen that is *present in early pregnancy and absent at about two months post-partum.* None of the references cited by the Examiner, either alone or in combination, teach pregnancy associated antigens or antibodies thereto that are both present early and absent about two months post-partum, as required by the claims. Without such a teaching, the claims can neither be anticipated nor rendered obvious.

VIII. ARGUMENT

A. *The Claims are Not Anticipated by Roberts et al. (1995)*

Claims 1, 3, 5, 6 and 14 were finally rejected under 35 U.S.C. §102(b) as anticipated by Roberts *et al.* (1995) (Exhibit A). The examiner characterizes the reference as teaching evaluation of maternal serum concentrations for PAGs, and correlating measurement to pregnancy in cattle and sheep. However, Roberts *et al.* (1995) does not teach PAGs meeting the claim limitations. Claim 1, upon which each of the remaining claims depends, reads as follows:

1. A method for detecting pregnancy in a bovine animal comprising:
 - (a) obtaining a sample from said animal; and
 - (b) contacting said sample with an antibody that binds immunologically to at least one pregnancy associated antigen (PAG), wherein said PAG is present in early pregnancy and absent at about two months post-partum; and

(c) detecting said PAG bound to said antibody;

whereby the presence of said PAG in said sample indicates that said animal is pregnant.

Roberts *et al.*, however, does not disclose a PAG that is “present in early pregnancy and absent at about two months post-partum”. For example, in the last two sentences of the first full paragraph on page 233 of Roberts *et al.*, it is indicated that the disclosed PAG, “PAG-1”, had an apparent long half-life and that “[b]ecause concentrations at term may be well above 1 µg/ml, *it requires at least 3 months for levels to drop back to threshold values* (Fig. 1), and cows are customarily bred within 2 to 3 months after calving.” Emphasis added. A review of the referenced Fig. 1, which is also given in Zoli *et al.* (1992), demonstrates this same phenomenon, showing that mean bPAG levels were above 1 ng/ml at 80 days post-partum. A review of the Zoli *et al.* abstract, indicates that the undetectable level for serum bPAG levels was less than 0.20 ng/ml. It is thus evident that Roberts *et al.* does not teach PAGs meeting the claim limitations.

Based on an earlier telephonic interview in the case, it is believed by Applicants that the position of the Examiner relies, at least in part, on the belief that the PAG taught by Roberts *et al.* could potentially be viewed as within the scope of the claims because of the use of the term “about two months post-partum”. Emphasis added. However, the PAG described by Roberts *et al.* is indicated to be present at least *three* months post-partum. This cannot be said to be “about two months”, as this is a figure 50% greater than two months. The term “about” is well known to those of skill in the art and does not allow for such a discrepancy. For example, the Encarta™ online dictionary (<http://dictionary.msn.com>), gives the meaning of the relevant usage of the word as a preposition as “approximately: close to in number, time, or degree.” Exhibit F. The relevant definition of “about” from the online version of the Merriam Webster’s Collegiate

Dictionary™ (<http://www.m-w.com>) is “reasonably close to”. Exhibit G. Therefore, an interpretation of “about two months” to include three months simply does not fit the meaning of the term as it is understood by those of skill in the art. Without teaching such a PAG, the cited reference cannot anticipate the claims.

Appellants finally note that the Examiner has provided no basis for concluding that Roberts *et al.* teaches the PAGs required by the instant claims other than to make the conclusory allegation that the “prior art teaches such PAGs.” For example, the one-paragraph rejection contained in both the first and the final Office Action states that the PAG in Roberts *et al.* was “expressed just prior to implantation until term (~145 days in sheep, ~280 days in cattle).” However, this is irrelevant to the claimed subject matter, which specifies immunologic detection of at least one PAG that is “present in early pregnancy and absent at about two months post-partum.” What is relevant, and is not even alleged by the Examiner, is that the PAG be *absent about two months post-partum*. The above-cited portions of Roberts *et al.* clearly indicate it is not. The rejection by the examiner thus fails on its face to present any basis that could be used to reject the claims.

It is the burden of the Examiner, in a rejection under 35 U.S.C. § 102(b), to show that each and every element as set forth in the claim is found in the prior art. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). The identical invention must be shown in as complete detail as is contained in the claim. *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989). To the extent that unexpressed inherent characteristics form the basis of an anticipation rejection, it must be shown that these characteristics necessarily flow from the disclosure. *Continental Can Co. USA v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991) (“To

serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill.") The Examiner has clearly failed to meet this burden. Accordingly, the rejection must fail.

In view of the foregoing, reversal of the rejection is respectfully requested.

B. The Claims are Not Anticipated by Zoli et al. (1992)

Claims 1, 3, 5, 6 and 13 were finally rejected under 35 U.S.C. §102(b) as anticipated by Zoli et al. (1992) (Exhibit B). The reference was cited as disclosing a double-antibody RIA for BoPAGs, and measuring BoPAG levels during pregnancy in cows.

Appellants again note that the cited reference fails to teach PAGs meeting the claim limitations. For example, attention is drawn to the Abstract of Zoli, which indicates that peripheral serum bPAG concentrations were 1.44 +/- 1.08 ng/ml at day 90 post partum. Thus the mean bPAG concentration of at least 0.36 ng/ml at 90 days post-partum was nearly twice the indicated undetectable level of <0.20 ng/ml that was given in Zoli et al. At most, the level was 2.52 ng/ml, or more than 10 times the undetectable level. As admitted in both the first and the final Office Action, the undetectable concentration was not reached until day 100 +/- 20 pp.

The problem created by the late presence of the Zoli et al. PAG is acknowledged on page 89 of Zoli et al., where it is stated that "[o]verall, the presence of bPAG in sera for nearly 100 days pp constitutes a problem for subsequent diagnosis of pregnancy by this method if rebreeding occurs less than 80 days pp." Thus, again, the mean bPAG levels detected by Zoli et al. did not drop below undetectable levels until more than 3 months post-partum. Even if one were to assume that the Zoli et al. bPAG was present at least 80 days post-partum, this figure

cannot be considered to be "about two months" As described above, the term "about" has a well known meaning in the art of close or reasonably close to a referenced figure. However, 80 days is approximately 33% longer than two months. This is cannot reasonably be construed to be "close" in time to two months. As such, the absence of this element from the prior art means that an anticipation rejection will not stand.

Reversal of the rejection is thus respectfully requested.

C. Claims 4, 7 and 8 are Not Obvious Under 35 U.S.C. §103 over Roberts et al. (1995) or Zoli et al. (1992) in view of Sasser et al. (1989)

Claims 4, 7 and 8 were finally rejected as obvious over Roberts *et al.* (1995) or Zoli *et al.* (1992) in view of Sasser *et al.* (1989) (Exhibit C). In particular, Roberts *et al.* and Zoli *et al.* were cited as above and Sasser *et al.* was cited as teaching use of saliva, milk or urine as samples to PAG.

As indicated above, regardless of what Sasser *et al.* may or may not disclose regarding means of sampling PAGs, it clearly does not address the material element of "at least one pregnancy associated antigen (PAG), wherein said PAG is present in early pregnancy and absent at about two months post-partum." The Examiner has not alleged otherwise. Further, Sasser *et al.* indicates that the PAG studied, "PSPB," was previously found to "remain[] in the serum for a considerable time after parturition in cows" and that "[t]his long half-life poses a problem for pregnancy testing in re-mated post-partum cows." Sasser *et al.* at page 111, third full paragraph. Therefore, the Examiner has not shown that the prior art teaches or suggests all of the limitations of the claims, as is required under 35 U.S.C. § 103. See, e.g., *In re Vaeck* 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). Absent such a teaching, one of skill in the art would lack a

reasonable expectation of success in arriving at the claimed invention. Therefore, the claims cannot be considered obvious.

In view of the foregoing, Appellants respectfully request reversal of the rejection.

D. Claims 30-34 are Not Obvious Under 35 U.S.C. §103 over Roberts et al. (1995) and Zoli et al. (1992) in view of Xie et al. (1997) and Gerrie et al. (1986)

Claims 30-34 remain rejected over Roberts *et al.* (1995) and Zoli *et al.* (1992) in view of Xie *et al.* (1997) (Exhibit D) and Gerrie *et al.* (1986) (Exhibit E). Roberts *et al.*, Zoli *et al.* and Xie *et al.* are cited as above and Gerrie *et al.* is cited as teaching an ELISA for PAG.

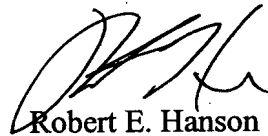
In response, Appellants first point out that the human pregnancy-associated $\alpha 2$ -glycoprotein is completely unrelated to the PAGs being discussed here. Thus, to the extent that the examiner is attempting to extrapolate more from Gerrie *et al.* than an ELISA based-assay for diagnosing pregnancy, Appellants submit that such is not merited. In fact, it should be pointed out that a variety of different assay formats may be employed according to the present invention, including but not limited to ELISA, RIA, Western blot, dot-blot and lateral flow technology.

More to the point, and irrespective of what Gerrie may or may not disclose regarding human PAGs, it clearly does not address the material element of "at least one pregnancy associated antigen (PAG), wherein said PAG is present in early pregnancy and absent at about two months post-partum." That is, Gerrie *et al.*, just like Roberts *et al.*, Zoli *et al.* and Xie *et al.*, provides no indication that such PAGs even exist. For example, Xie *et al.* is directed to sheep (ovine) PAGs that are related to the bovine PAG-1 discussed in Roberts *et al.* Abstract, Xie *et al.* It, therefore, remains pure hindsight to argue that any of these references can suggest, with sufficient motivation or the requisite likelihood of success, the currently claimed invention. Thus, in light of the foregoing, Appellants respectfully request reversal of the rejection.

IX. CONCLUSION

It is respectfully submitted, in light of the above, none of the pending claims are anticipated under 35 U.S.C. §102(b) or obvious under 35 U.S.C. §103(a). Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,



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Date: August 15, 2002

**APPENDIX 1: APPEALED CLAIMS PRIOR TO ENTRY OF
AMENDMENT UNDER 37 C.F.R. § 1.116**

1. A method for detecting pregnancy in a bovine animal comprising:
 - (a) obtaining a sample from said animal; and
 - (b) contacting said sample with an antibody that binds immunologically to at least one pregnancy associated antigen (PAG), wherein said PAG is present in early pregnancy and absent at about two months post-partum; and
 - (c) detecting said PAG bound to said antibody;

whereby the presence of said PAG in said sample indicates that said animal is pregnant.
2. The method of claim 1, wherein said PAG is selected from the group consisting of PAG2, PAG4, PAG5, PAG6, PAG7 and PAG9.
3. The method of claim 1, wherein said sample is saliva, serum, blood, milk or urine.
4. The method of claim 3, wherein said sample is saliva.
5. The method of claim 3, wherein said sample is serum.
6. The method of claim 3, wherein said sample is blood.
7. The method of claim 3, wherein said sample is milk.
8. The method of claim 3, wherein said sample is urine.
10. The method of claim 1, wherein said detection comprises detection of bovine PAG (BoPAG) 2, BoPAG4, BoPAG5, BoPAG6, BoPAG7, BoPAG9, BoPAG 7v; BoPAG9v; BoPAG 15; BoPAG 16; BoPAG 17; BoPAG 18; BoPAG 19; BoPAG 20 or BoPAG 21 with polyclonal antisera.

11. The method of claim 1, wherein said detection comprises detection of bovine PAG (BoPAG) 2, BoPAG4, BoPAG5, BoPAG6, BoPAG7, BoPAG9, BoPAG 7v; BoPAG9v; BoPAG 15; BoPAG 16; BoPAG 17; BoPAG 18; BoPAG 19; BoPAG 20 or BoPAG 21 with a monoclonal antibody preparation.
12. The method of claim 9, wherein said detection comprises ELISA.
13. The method of claim 9, wherein said detection comprises RIA.
14. The method of claim 9, wherein said detection comprises Western blot.
30. The method of claim 1, further comprising detecting a second PAG in said sample.
31. The method of claim 30, further comprising detecting a third PAG in said sample.
32. The method of claim 12, wherein said ELISA is a sandwich ELISA comprising binding of a PAG to a first antibody preparation fixed to a substrate and a second antibody preparation labeled with an enzyme.
33. The method of claim 32, wherein said enzyme is alkaline phosphatase or horseradish peroxidase.
34. The method of claim 32, wherein said first antibody preparation is monoclonal.

**APPENDIX 2: APPEALED CLAIMS FOLLOWING ENTRY OF
AMENDMENT UNDER 37 C.F.R. § 1.116**

1. A method for detecting pregnancy in a bovine animal comprising:
 - (a) obtaining a sample from said animal; and
 - (b) contacting said sample with an antibody that binds immunologically to at least one pregnancy associated antigen (PAG), wherein said PAG is present in early pregnancy and absent at about two months post-partum; and
 - (c) detecting said PAG bound to said antibody;

whereby the presence of said PAG in said sample indicates that said animal is pregnant.
2. The method of claim 1, wherein said PAG is selected from the group consisting of PAG2, PAG4, PAG5, PAG6, PAG7 and PAG9.
3. The method of claim 1, wherein said sample is saliva, serum, blood, milk or urine.
4. The method of claim 3, wherein said sample is saliva.
5. The method of claim 3, wherein said sample is serum.
6. The method of claim 3, wherein said sample is blood.
7. The method of claim 3, wherein said sample is milk.
8. The method of claim 3, wherein said sample is urine.
10. The method of claim 1, wherein said detection comprises detection of bovine PAG (BoPAG) 2, BoPAG4, BoPAG5, BoPAG6, BoPAG7, BoPAG9, BoPAG 7v; BoPAG9v;

BoPAG 15; BoPAG 16; BoPAG 17; BoPAG 18; BoPAG 19; BoPAG 20 or BoPAG 21 with polyclonal antisera.

11. The method of claim 1, wherein said detection comprises detection of bovine PAG (BoPAG) 2, BoPAG4, BoPAG5, BoPAG6, BoPAG7, BoPAG9, BoPAG 7v; BoPAG9v; BoPAG 15; BoPAG 16; BoPAG 17; BoPAG 18; BoPAG 19; BoPAG 20 or BoPAG 21 with a monoclonal antibody preparation.
12. The method of claim 1, wherein said detection comprises ELISA.
13. The method of claim 1, wherein said detection comprises RIA.
14. The method of claim 1, wherein said detection comprises Western blot.
30. The method of claim 1, further comprising detecting a second PAG in said sample.
31. The method of claim 30, further comprising detecting a third PAG in said sample.
32. The method of claim 12, wherein said ELISA is a sandwich ELISA comprising binding of a PAG to a first antibody preparation fixed to a substrate and a second antibody preparation labeled with an enzyme.
33. The method of claim 32, wherein said enzyme is alkaline phosphatase or horseradish peroxidase.
34. The method of claim 32, wherein said first antibody preparation is monoclonal.

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about



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a·bout [ə bówt] **CORE MEANING:** a grammatical word that refers to different sides or aspects of something from some point of orientation • *(prep)* a book about a dog
• *(adv)* There's a lot of laziness about.

1. preposition

in connection with: in connection with or relating to • *think about problems*

2. preposition

approximately: close to in number, time, or degree • *inviting about 15 people*

3. preposition

doing or attending to: with or in an activity
• *go about your business*

4. preposition

close by: placed, located, or happening close by or around • *frantic activity going on all about us*

5. preposition

around: around or on a place or person • *a red scarf about her neck*

6. adverb preposition

in various places: positioned here and there
• *scattered about the house*

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- *scattered about the house*

7. *adverb preposition*

in different directions: from place to place in different directions or in no particular direction • *children running about everywhere*

8. *adverb*

in circulation: available or in circulation

- *there was never much money about*

9. *adverb*

into a reversed position: in or to the opposite direction • *the wrong way about*

10. *adverb*

all around: on every side of or all the way around "He proceeded to the banks of the Hudson, and looked about among the vessels." Jules Verne *Around the World in 80 Days* (1873)

11. *adverb*

used as intensifier: used to emphasize a statement, usually when expressing impatience or anger (*informal*) • *Well, it's about time you showed up!*

12. *adverb*

NAUTICAL to opposite tack: on or to the opposite tack

[Old English *onbūtan* "on or around the outside of," from *on* (see on) + *būtan* (see but)]

be about to be on the point of doing something • *The game was about to start.*

be what something or somebody is (all) about to be what something or somebody involves or has as a purpose (*informal*)

not about to used to emphasize that somebody is certainly not going to do something (*informal*) • *I'm not about to apologize!*

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un-abridged \ən-ə-'brīdʒ
adj : being the most complete
in its class

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is just the
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about[1,adverb]
about[2,preposition]
about[3,adjective]

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Main Entry: ¹**about** ㉔

Pronunciation: ə- 'bawt

Function: *adverb*

Etymology: Middle English, from Old English *abutan*, from ¹a- +
butan outside -- more at **BUT**

Date: before 12th century

1 a : reasonably close to <*about* a year ago> **b** : **ALMOST** <*about*
starved> **c** : on the verge of -- usually used with *be* and a following
infinitive <*is about* to join the army> -- used with a negative to
express intention or determination <*not about* to quit>

2 : on all sides : **AROUND**

3 a : in rotation **b** : around the outside

4 : **HERE AND THERE**

5 : in the vicinity : **NEAR**

6 : in succession : **ALTERNATELY** <*turn about* is fair play>

7 : in the opposite direction <*face about*> <*the other way about*>

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\&r\ as ur/er in further
\a\ as a in ash
\A\ as a in ace
\ä\ as o in mop
\au\ as ou in out
\ch\ as ch in chin

\e\ as e in bet
\E\ as ea in easy
\g\ as g in go
\i\ as i in hit
\I\ as i in ice
\j\ as j in job
\[ng]\ as ng in sing
\O\ as o in go

\o\ as aw in law
\oi\ as oy in boy
\th\ as th in thin
\[th]\ as th in the
\ü\ as oo in loot
\u\ as oo in foot
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GLYCOPROTEINS OF THE ASPARTYL PROTEINASE GENE FAMILY SECRETED BY THE DEVELOPING PLACENTA

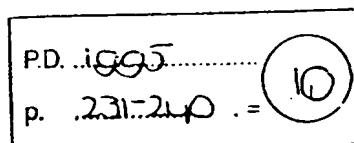
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SUMMARY

Pregnancy in cattle and sheep can be diagnosed by the presence of placentally-derived antigens (pregnancy-associated glycoproteins or PAG-I) in maternal serum soon after implantation begins at about Day 20 following conception. Molecular cloning of their cDNA has revealed that PAG-I belong to the aspartic proteinase gene family and have about 50% amino acid sequence identity to pepsin. However, critical amino acid substitutions at the active site regions suggest that both bovine and ovine PAG-I are enzymatically inactive. PAG-I expression has been shown by *in situ* hybridization and immunocytochemistry to be localized to the trophoblast binucleate cells, which invade maternal uterine endometrium during implantation. The glycoproteins are concentrated in dense cytoplasmic granules that are discharged after the binucleate cells have migrated to the maternal side of the placental barrier. We suggest, therefore, that the PAG-I might have an endocrine function either as carriers of other bioactive peptides or by acting as hormones themselves. Recently screening of placental libraries with nucleic acid probes has identified additional cDNA that are very abundant and code for polypeptides (PAG-2 and PAG-3) related to, but antigenically and structurally distinct from PAG-I described above. These molecules have sequences of amino acids at their catalytic centers that are consistent with their being potentially functional proteinases but their role during pregnancy, like that of PAG-I, is unclear.

Aspartic Proteinases: Structure, Function, Biology, and Biomedical Implications
Edited by Kenji Takahashi, Plenum Press, New York, 1995

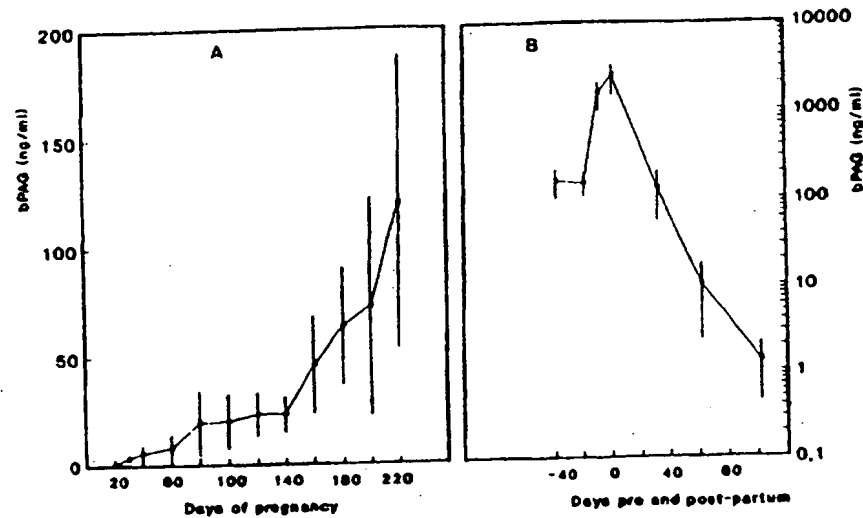


Figure 1. Profile of the concentrations (Mean \pm SD) of bPAG in serum of 20 cows bled from Day 20 of pregnancy until 100 days after parturition. Note the linear (A) and the logarithmic (B) scales of the Y axis respectively from 20 to 220 days of gestation and from Day 40 prepartum to Day 80 postpartum. From Zoli *et al.* (10) with permission.

PAG: A BRIEF HISTORY

Pregnancy in humans can be detected soon after the conceptus begins to implant by the presence of a placentally-produced hormone-human chorionic gonadotrophin (hCG)-first in the maternal bloodstream and a little later in urine (1). However, CG expression in gestation is a unique feature of primates and equids (2,3) and, as a consequence, a simple, unequivocal serum test for pregnancy based on CG is not available for other mammals, including the economically valuable farm species. In 1982 Butler *et al.* (4) reported the partial purification and characterization of a pregnancy-specific protein (PSP-B) in cattle. They first raised an antiserum against antigens in homogenates of placental membranes and used tissue and blood from nonpregnant cattle to adsorb out immunoglobulins not specific to pregnancy. The resulting antiserum was employed to monitor the partial purification of PSP-B, which was initially reported to have a molecular weight of 47,000 to 53,000 and a pI of \sim 4.0. Over the next decade PSP-B was never thoroughly characterized, although a range of molecular weights was published (4-6).

In 1991 Zoli *et al.* (7) reported the purification of a pregnancy-associated glycoprotein (PAG, but here called PAG-1) from bovine placental tissue. The methodology was similar to that of Butler *et al.* (4) in that an antiserum was first generated against placental extracts from which antibodies against common maternal antigens had been removed by immunoadsorption. This reagent was then used to track PAG-1 purification through a series of chromatographic steps that led to the isolation of an acidic glycoprotein product of M_r \sim 67,000. It is now clear that PSP-B and PAG-1 are identical products (8).

The presence of PAG-1 (or PSP-B) in blood serum has provided the basis of a potentially useful pregnancy test in cattle. The antigen generally becomes detectable by about Day 20 post-breeding and all pregnant cows appear to have measurable levels by Day 24 (Fig. 1) (5,9,10). This period coincides with the time that the trophoblast attaches

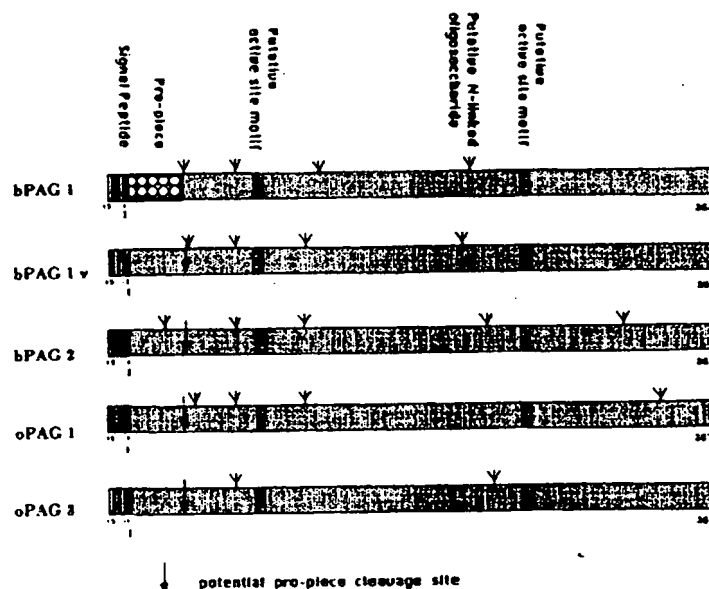


Figure 2. Diagram showing the predicted structures of the five PAG polypeptides whose cDNA have so far been cloned. Of these, only bPAG-1 has been purified and its NH₂-terminal amino acid sequence confirmed. Data indicate that a pro-peptide has been removed at position +38. For each PAG the putative signal sequence (bars); potential sites of attachment of carbohydrate chains to Asn residues () and the polypeptide regions associated with the catalytic center are shown. The lengths of the signal sequences in each case were 15 residues. The lengths of the pre-proteins ranged from 367 (oPAG-1) to 361 (bPAG-2 and oPAG-3).

itself firmly to the uterine wall and when placentation begins (11). Concentrations of the antigen rise gradually during gestation and peak just prior to parturition. The antiserum raised against PSP-B (PAG-1) has since been used to monitor pregnancy in sheep (12), goats (13), deer (14) and wood bison (15).

The reason for the relatively late appearance of PAG-1 in cattle, relative to hCG in women, probably relates to the fact that implantation is initiated correspondingly later in cows, and PAG-1 is produced exclusively by the invading trophoblast binucleate cells (16,17). Although a very early pregnancy test is desirable in the cattle industry, the blood test for PAG-1 can be performed earlier than the presently used alternative (rectal palpation). One major shortcoming of the blood test, however, lies in the apparent long half-life (~9 days) of PAG-1 in maternal blood. Because concentrations at term may be well above 1 µg/ml, it requires at least 3 months for levels to drop back to threshold values (Fig. 1), and cows are customarily bred within 2 to 3 months after calving.

THE cDNA CLONING OF PAG

The antiserum generated by Zoli *et al.* (7) was used to screen cDNA libraries prepared from mid-pregnant placental tissues of cattle and sheep (16). The cDNA so isolated were approximately 1.7 kbp in length and coded for polypeptides of 380 and 382 amino acids in cattle and sheep respectively (Fig. 2). Each polypeptide had a predicted signal sequence of

	bPAG 1	bPAG 1v	bPAG 2	oPAG 1	oPAG 3	hPepA	hCatD	hCatE
bPAG 1	100							
bPAG 1v	86.5	100						
bPAG 2	57.9	58.5	100					
oPAG 1	72.0	73.8	58.7	100				
oPAG 3	59.7	58.9	64.7	59.8	100			
hPepA	49.5	50.4	49.1	51.2	49.9	100		
hCatD	37.5	38.8	37.4	41.4	41.7	43.3	100	
hCatE	42.1	41.8	43.7	41.8	44.4	53.5	51.6	100

Figure 3. Primary amino acid sequence similarities among the various PAG and selected aspartyl proteinases. The sequences for human pepsinogen A (hPepA), human cathepsin D (hCatD) and human cathepsin E (hCatE) were derived from Sogawa *et al.* (22), Faust *et al.* (23) and Azuma *et al.* (24).

15 residues and, in the case of the bovine PAG where an amino terminal sequence of the purified glycoprotein had already been obtained (7), it was clear that a pro-piece of 38 residues had also been removed.

Ovine PAG-1 has yet to be isolated and chemically sequenced. Most surprisingly the PAG clearly belonged to the aspartic proteinase gene family (Fig. 3) and especially resembled pepsin, where the extent of sequence identity was close to 50%. Although there are considerable stretches of sequence where PAG and pepsin sequences differ markedly (Fig. 4), examination of the corresponding nucleotide sequence of these genes indicates that many of these differences arose from sudden frameshifts. Despite the resemblance to pepsin, the purified bPAG-1 product had no detectable proteolytic activity towards denatured hemoglobin, yet it bound tightly to immobilized pepstatin and so presumably had an intact substrate-binding cleft (16).

Examination of the normally conserved regions in the amino terminal and carboxyl terminal lobes, which together constitute the catalytic centers of aspartic proteinases revealed yet further surprises. Ovine PAG-1 (oPAG-1) lacked one of the invariant aspartyl residues considered essential for catalysis and could not, therefore, be active. In contrast, bPAG-1 had an alanine substituted for a normally invariant glycine residue (Fig. 5). A molecular modeling study performed in Dr. Jordan Tang's laboratory has indicated that the presence of the relatively bulky methyl side chain of alanine would probably cause considerable displacement of the water molecule that resides symmetrically between the two adjacent aspartic acid residues (Fig. 6). Because this water molecule is involved in peptide bond cleavage, there is good reason to suppose that neither ovine nor bovine PAG can be catalytically active. Consequently, the removal of the pro-piece from bPAG-1 is probably not achieved autocatalytically. More likely some other proteinase is responsible.

If the PAG-1 molecules have any function, therefore, it seems unlikely that it is in proteolysis. Two alternative roles, both of which capitalize on the apparently intact substrate-binding cleft, immediately suggest themselves. One is that the PAG-1 bind and transport bioactive peptides, possibly in order to neutralize their effects or to extend the half-lives of such molecules in much the same way as specific-binding proteins interact with the insulin-like growth factors (18). Alternatively, the PAG-1 may themselves be hormones which bind to specific amino acid sequences on receptor molecules.

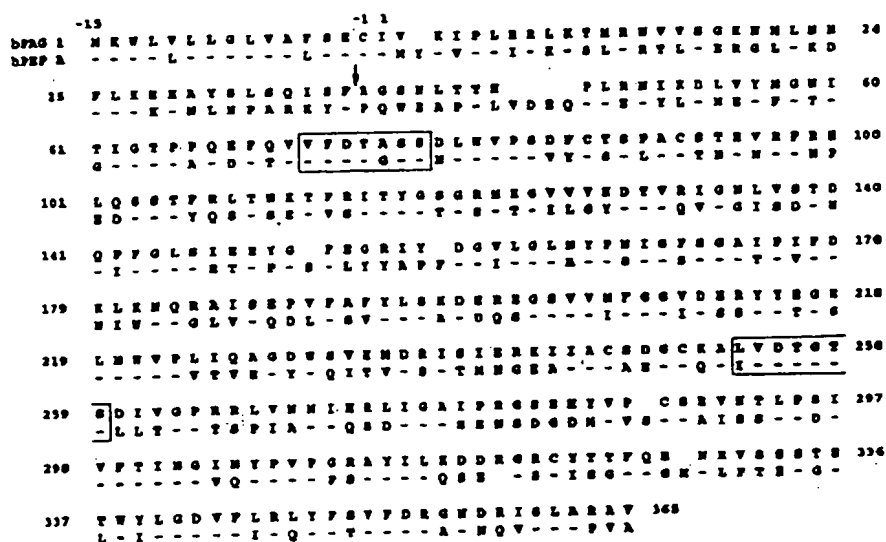


Figure 4. Comparison of the amino acid sequences of bPAG-1 and human pepsinogen A. Dashes in the hPepA sequence indicate identity. The sequences in the two catalytic site regions are shaded and boxed. Gaps have been placed to maximize alignments.

EXPRESSION OF PAG-1 DURING PREGNANCY

Immunocytochemical studies have shown that PAG-1 in both cattle and sheep is localized to the binucleate cells in the outer epithelial layer (trophoblast) of the placenta (Fig. 7).

These cells, which probably arise by endomitosis from precursor mononucleate cells in the same epithelium, start to appear just prior to the time that the pre-placenta (trophoblast) attaches to the uterine wall (around Day 17 in cattle). They constitute the invasive components of the placenta of ruminant species, and, once formed, migrate from the trophoblast and fuse with maternal uterine epithelial cells. At this stage the dense secretory granules discharge their contents towards the capillaries that feed the basolateral surface of the uterine epithelium. The PAG-1 co-localize to these dense granules with placental lactogen (19-20), which is an established hormone, thus reinforcing the notion that they may have an endocrine function.

The PAG-1 mRNA is expressed abundantly from the time binucleate cells first form, just prior to implantation, until term (~145 days in sheep, ~280 days in cattle). In this regard their expression differs markedly from that of another trophoblast-specific product of ruminant species, the interferon-tau (IFN- τ). The latter is localized to the mononucleate cells of the trophoblast and is expressed for only a few days prior to the beginning of implantation (21).

IDENTIFICATION OF cDNA FOR ADDITIONAL PAG

Recently nucleic acid screening of the bovine cDNA libraries under low conditions of stringency has revealed cDNA for additional PAG molecules. The first to be characterized

PROTEIN	N-TERMINUS	C-TERMINUS
Bovine PAG-1	FDT <u>A</u> SS	VDTGTS
Ovine PAG-1	FDTGSS	VDTGTS
Papain (feline)	FDTGSS	VDTGTS
Chymotrypsin (bovine)	FDTGSS	VDTGTS
Renin (mouse)	FDTG <u>S</u> A	VDTG <u>S</u> S
Cathepsin D (human)	FDTGSS	VDTGTS
Cathepsin E (human)	FDTGSS	VDTGTS
Consensus	FDTGSS	VDTGTS

Figure 5. Alignment at the active site residues found in a variety of aspartyl proteinases. The open boxes denote substitutions that do not alter enzymatic activity. Amino acid substitutions that are likely to render the enzyme inactive are underlined. Data for aspartyl proteinase sequences are derived from Evers *et al.* (25), Tang and Wong (26), Hidaka *et al.* (27), Miyazaki *et al.* (28) and the references for Figure 3.

encoded a protein with 87% amino acid sequence identity to bPAG-1. It has been named bPAG-1_{ov} and is not discussed further here. A second highly abundant cDNA, which represents a 376 amino acid polypeptide (PAG-2) with only 58% amino acid sequence identity to bPAG-1, was isolated by a similar screening strategy (Fig. 2). Curiously, the sequences comprising the catalytic center are consistent with PAG-2 being a potentially active aspartyl proteinase (Fig. 5), although such proteolytic activity remains to be demonstrated. This laboratory is currently producing bPAG-2 in *E. coli* by recombinant techniques. Provided the molecule can be successfully renatured and activated, we should soon be able to test the ability of PAG-2 to cleave a range of substrates.

Nucleic acid screening of the ovine cDNA library has revealed the transcripts for yet another PAG produced by the ovine placenta that is sufficiently distinct in sequence from oPAG-1 and bPAG-2 to constitute yet another subtype (oPAG-3). This molecule also possesses an apparently intact catalytic center (Fig. 5).

Southern genomic blot analysis of bovine DNA with exon-specific cDNA probes strongly suggests that there are many (probably at least ten) PAG-related genes (data not shown). Although it is unclear whether these genes are expressed in placenta or elsewhere, it seems possible that the PAG family of proteins may be extensive.

POST-TRANSLATIONAL PROCESSING OF PAG-1

After pro-piece removal, the polypeptide portion of PAG-1 has a molecular weight of about 37,000, yet when purified PAG-1 is analyzed on SDS-polyacrylamide gels it migrates with an apparent M_r close to 70,000. Part of this mass is undoubtedly derived from carbohydrate as PAG-1 is known to be a glycoprotein with at least 10% by weight neutral sugar (7). Treatment of PAG-1 with N-glycanase to remove oligosaccharides linked to asparagine residues reduces the apparent M_r to about 60,000 (data not shown), and biosynthetic studies carried out on cultured explants of placenta in presence of tunicamycin, an inhibitor of N-glycosylation, have shown a similar modest reduction in PAG-1 size. Whether this "extra" mass of PAG-1 is due to O-linked carbohydrate or to some other form of post-translational modification is unclear. It does not appear to result from any unusual physical properties of the polypeptide itself, because, when synthesized as a glycosylated recombinant product in CHO or Cos-1 cells, its mobility in SDS-gels is consistent with an

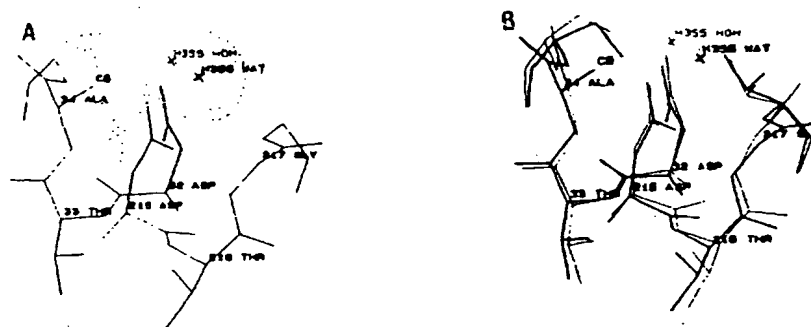


Figure 6. Computer modeling of the highly conserved regions of PAG, homologous to the active sites of aspartic proteinases. (A) Overlay of the modeled bPAG structure with the active site of porcine pepsin (29). Bold lines depict the structure of the bPAG residues homologous to the active site of porcine pepsin, with α -carbons labeled according to pepsin numbering. Thin lines are the active site of the crystal structure of porcine pepsin. The water molecule from the PAG model is labeled as H355 WAT, and the solvent from the crystal structure of porcine pepsin is labeled as H355 HOH. (B) Computer model of the elements of bPAG homologous to the active site of pepsin. The "active-site" Asp-32 and Asp-215 (pepsin numbering) are plotted with bold lines. The van der Waals surface of water molecule H355 WAT and Ala-34 from the PAG model are depicted as stippled areas. The native position of H355 HOH from the crystal structure of porcine pepsin is drawn and labeled with light lines. The native position of the solvent is altered by the presence of the methyl group of the Ala-34 β -carbon, labeled CB. From Xie *et al.* (16) with permission.

M_r of 43,000. It seems likely, therefore, that binucleate cells of the placenta process PAG in some unusual manner.

Even though PAG is first synthesized as a ~70K product in explant cultures and that form tends to predominate in the tissues, smaller sized molecules accumulate with time, particularly in the medium (Fig. 8). Recent pulse-chase experiments with labeled amino acids have clearly established that the 70K form precedes the formation of the 47K and 53K forms. To date the amino terminal sequences of these secondary products have not been obtained and so the nature of the processing events remains unclear. Nevertheless their presence probably explains the range of sizes that has been reported for PAG-I (or PSP-B) in the years following its first description in 1982.

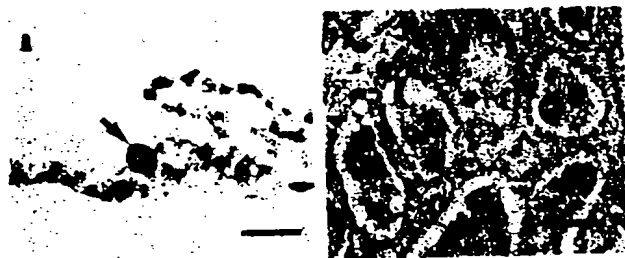


Figure 7. Immunohistochemical localization for PAG in trophoblast and placenta. bPAG staining was detected in binucleate cells (indicated by arrow) within trophoblast of Day 18 bovine conceptus (A) and in bovine placenta of midgestation fetal cotyledonary villi (B) that interdigitate with maternal uterine tissue. (Bars = 50 μ m) From Xie *et al.* with permission.

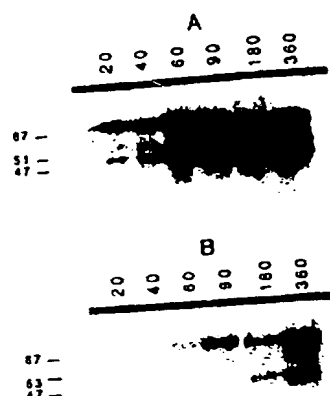


Figure 8. Time course of oPAG biosynthesis in ovine placental explants *in vitro*. Trophoblast tissue from a Day 25 conceptus was incubated for the time periods (in min) shown above the lanes. Immunocomplexes from tissue extract (A) and medium (B) were analyzed in 10% polyacrylamide gels containing SDS and radioactive polypeptides were detected by fluorography. Molecular weights on the left ($\times 10^{-3}$) were calculated from standards electrophoresed on each side of the gel. From Xie *et al.* (16) with permission.

DISCUSSION

The realization that the placenta-specific antigens PAG-1 from cattle and sheep were related in sequence to aspartyl proteinases, and especially to pepsin, but lacked functional catalytic centers was surprising, yet consistent with the hypothesis that members of proteinase gene families need not necessarily display their function through the hydrolysis of peptide bonds. It is possible, of course, that PAG-1 is expressed promiscuously in placenta as a functionless product, but we prefer the alternative hypothesis that it uses its apparently intact substrate-binding cleft to associate specifically with some other protein or polypeptide. The presence of other abundant transcripts representing additional aspartyl proteinases resembling PAG, yet possessing apparently intact catalytic centers, constitutes a further puzzle. It remains to be determined whether these molecules are catalytically active and, even if they are, whether their physiological function is that of proteinases.

ACKNOWLEDGMENTS

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Radioimmunoassay of a Bovine Pregnancy-Associated Glycoprotein in Serum: Its Application for Pregnancy Diagnosis¹

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ABSTRACT

A sensitive and specific double-antibody RIA for a bovine pregnancy-associated glycoprotein (bPAG) is described. The limit of detection was 0.2 ng/ml. The assay was specific for bPAG in that pituitary and placental gonadotropic hormones and other placental or serum proteins assayed in serial dilutions did not cross-react. The RIA allowed measurement of bPAG in placental extracts, fetal serum, fetal fluids, and serum or plasma of pregnant cows. About 20% of unbred heifers and nonpregnant cows had detectable levels ranging from 0.30 ± 0.09 to 0.50 ± 0.17 ng/ml (mean \pm SD), and 15% of bull sera showed higher concentrations (3.01 ± 1.73 ng/ml) of bPAG or bPAG-like protein. Variations among animals was observed in fetal serum bPAG concentrations. Bovine PAG was detected in maternal peripheral blood at Day 22 of pregnancy (mean \pm SD, 0.38 ± 0.13 ng/ml) in some animals and at Day 30 in all pregnant cows. Peripheral serum bPAG levels increased progressively to 3.60 ± 1.73 ng/ml (mean \pm SD) at Day 30 of pregnancy, to 24.53 ± 8.81 ng/ml at Day 120, and to 1551.91 ± 589.68 ng/ml at Day 270. Peak concentration of bPAG was 2462.42 ± 1017.88 ng/ml and it occurred 1-5 days prior to parturition. After delivery, bPAG concentrations decreased steadily to 499.63 ± 267.20 ng/ml at Day 14 postpartum (pp), 10.12 ± 7.84 ng/ml at Day 60 pp, and 1.44 ± 1.08 ng/ml at Day 90 pp. The undetectable concentration (<0.20 ng/ml) was reached by Day 100 ± 20 pp.

An investigation undertaken in Holstein heifers, Holstein cows, and Hereford cows used as recipients for purebred Holstein embryos supplied evidence of the influence of breed of recipient and sex of fetuses on peripheral concentrations of bPAG.

A herd of 430 Holstein-Friesian heifers that had received transferred embryos were bled at Day 35 postestrus (pe) for measurement of bPAG. The bPAG was detected in 287 of 430 serum samples analyzed. By rectal palpation performed at Day 45 pe, 267 heifers with detectable levels of bPAG at Day 35 pe were confirmed to be pregnant as were 3 of 143 heifers previously diagnosed as not pregnant by RIA. These results suggest that detection of this placental-specific antigen in the serum could be used as a specific serological method for early pregnancy diagnosis in cattle from 28 days after breeding.

INTRODUCTION

During normal pregnancy in human and various animal species, a number of hormones and proteins either appear for the first time or are greatly increased in the maternal circulation. Many of these hormones and proteins are of fetal-placental origin rather than of maternal origin [1]. For example, hCG, first reported by Aschheim and Zondek [2], is present in peripheral blood and urine of pregnant women 6-10 days after conception [3]. Another trophoblastic protein, human placental lactogen (hPL), is detectable only from Week 9 of pregnancy [4]. Concentrations of hPL in maternal peripheral serum rise progressively during gestation, and the measurement of hPL is of diagnostic value in detecting and monitoring high-risk pregnancies [5, 6]. In domestic animals, several pregnancy proteins have also been re-

ported. Equine chorionic gonadotrophin (eCG) [7] currently is used for pregnancy diagnosis in mares [8]. Bovine placental lactogen (bPL), synthesized by trophoblastic binucleate cells [9], is detectable in the maternal peripheral blood after Day 110 of gestation [10].

During the past twenty years, several new placental proteins without any known hormonal activity have been isolated and studied [11]. In humans, the pregnancy-specific β 1-glycoprotein (SP₁) is detectable in the maternal serum as early as 6-14 days after conception and can be used for early detection of pregnancy [6]. In domestic ruminants, early trophoblastic proteins (ovine TP₁ and bovine TP₁) are involved in maintenance of the corpus luteum [12, 13]. However, trophoblastic proteins are not measurable in maternal serum [14]. Recently, a placental glycoprotein called pregnancy-specific protein B (PSPB) was isolated from fetal membranes [15, 16]. This protein is detectable in pregnant cow serum as early as 15 days after conception [17].

The present report describes the development of a sensitive and specific double-antibody RIA for a bovine pregnancy-associated glycoprotein (bPAG) previously isolated from fetal cotyledons [18] and its application for measurement of bPAG in fetal and maternal native blood and detection of pregnancy.

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MATERIALS AND METHODS

Materials

Antigen. Recently, four isoforms of bPAG of M_r 67 000 were highly purified from fetal cotyledonary tissue [18]. The pool of these four isoforms was used as antigen for rabbit immunization. The same pool of bPAG was dissolved (1 μ g/ml) in 0.025 M Tris buffer (pH 7.5) and stored at -20°C in 0.5-ml aliquots that were used when required either as standard or for labeling with ^{125}I .

Antiserum. Antisera against bPAG were raised in rabbits according to the method of Vaitukaitis et al. [19]. Rabbits received intradermal injections of 250 μ g of bPAG dissolved in 0.5 ml of distilled water and emulsified in an equal volume of Freund's complete adjuvant (Difco Labs., Detroit, MI). Rabbits received booster doses at 2-wk intervals with 250 μ g of bPAG in Freund's incomplete adjuvant and were bled 1 wk after the fourth injection and then every 2 wk. Sera thus obtained were tested for antibodies to bPAG.

Radiolabeled protein (tracer). The bovine ^{125}I -PAG was prepared according to the lactoperoxidase method of Thorrell and Johansson [20]. The reaction mixture was composed of 5 μ g of bPAG dissolved in 20 μ l of 0.025 M Tris buffer (pH 7.5), 25 μ l of 0.5 M phosphate buffer (pH 7.4), 2 mCi ^{125}I (Amersham Corp., Arlington Heights, IL), 10 μ l (1 μ g) of lactoperoxidase (Boehringer, Mannheim, FRG) and 30 μ l of 30% hydrogen peroxide (1:30 000 dilution). The reaction period was 8 min. Unreacted iodine was separated from bovine ^{125}I -PAG by gel filtration on a Sephadex G75 column (1 \times 30 cm; Pharmacia, Piscataway, NJ) by using 0.025 M Tris-0.01 M MgCl_2 (pH 7.5) containing 0.1% BSA as eluting buffer. The percentage of total radioactivity incorporated into bPAG was greater than 90%, and the resultant specific radioactivity, determined according to the self-displacement method of Roulston [21], was 0.401 ± 0.057 mCi/ μ g (means of five assays).

Double-antibody solid phase. An antiserum was raised in a sheep immunized against highly purified rabbit IgG. Blood samples were collected from the sheep at 20-day intervals, and the titers of the antiserum thus obtained were evaluated by a double immunodiffusion test. When the titer reached a sufficiently high value, the sheep was bled and the blood was allowed to clot. The sheep IgG was purified from serum according to the method of Harboe and Ingild [22] and then covalently coupled to microcrystalline cellulose (Merck, Rahway, NJ), which had been previously activated with cyanogen bromide. The activated cellulose antibody was used at a concentration of 2.5 mg/ml, and 1 ml was added in each assay tube except that containing total count (TC).

Buffer. All the reagents were dissolved in the same buffer: 0.025 M Tris + 0.01 M MgCl_2 (pH 7.5) containing 0.1% BSA and 0.01% neomycin sulfate.

Cotyledonary extracts, placental hormones and proteins, BSA, alpha-fetoprotein (AFP), and fetal fluids were assayed

in serial dilutions either to detect bPAG or to measure the cross-reactivity with the antiserum anti-bPAG.

Cotyledonary extracts. Bovine and ovine fetal cotyledons were extracted as described by Beckers et al. [23], and the 40–80% ammonium sulfate precipitate was dialyzed extensively against 0.01 M Tris (pH 7.5) buffer. The protein concentration was determined by the method of Lowry et al. [24].

Placental proteins, gonadotropic hormones, AFP, and BSA. The assay kit for human SP_1 was purchased from Diagnostic Products Corporation (Medico-service Benelux, Belgium); eCG (Folligon) was from Intervet (Boxmeer, The Netherlands). Bovine LH (bLH), bovine (bPL), and porcine FSH (pFSH) were purified in our laboratory [25, 26]. Highly purified BSA and AFP were purchased from Boehringer and Calbiochem-Behring Corporation (San Diego, CA), respectively.

Amniotic and allantoic fluids. Amniotic and allantoic fluids were collected by aspiration before fetal blood collection. Samples were centrifuged at $1\,500 \times g$ for 20 min and the supernatant was stored at -20°C until assayed.

Serum samples. Fetal blood samples were collected at the slaughterhouse from 90 fetuses; blood was taken from the umbilical vein of fetuses at early stages of pregnancy and by heart puncture of fetuses at later stages. Fetal ages were determined by crown-rump measurement [27]. Samples were also collected from the following groups of animals according to the following regimens: (1) 30 unbred heifers and 20 bulls; (2) a herd of 20 pregnant dairy and beef cows were bled once daily from the caudal vein from Day 20 to Day 35 postconception (pc) and then at 2-wk intervals until Day 100 postpartum (pp); (3) 14 Holstein heifers, 12 Holstein cows, and 9 Hereford cows carrying transferred purebred Holstein embryos were bled once daily from Day -20 prepartum to Day 2 pp (parturition = Day 0); and (4) a commercial herd of 430 Holstein-Friesian heifers carrying transferred embryos were bled at Day 35 postestrus (pe). Rectal palpations were performed at Day 45 pe to confirm pregnancy. Blood samples were allowed to clot and were centrifuged at $1\,500 \times g$ for 30 min. Sera obtained were stored at -20°C until assayed.

Methods

Standard curves. An aliquot of pure stock bPAG solution [18], 500 ng/ml, was diluted with assay buffer to give concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.20 ng/ml. Of each standard concentration, 0.1 ml was added to duplicate assay tubes. Buffer was added to zero standards (B_0 ; 0.2 ml) and tubes for evaluating non-specific binding (0.3 ml). To minimize nonspecific interference due to serum proteins, 0.1 ml of bPAG-free serum (a pool of unbred heifer or stallion sera) was added to all standard curve tubes except TC tubes. The stability of tracer as well as the repeatability and the sensitivity of the assay were tested over 28 standard curves within a 3-mo period.

Gonadotropic hormones, placental and fetal proteins, amniotic and allantoic fluids, pooled sera of full-term pregnant and nonpregnant cows, and placental extracts were assayed in serial dilutions.

Unknown samples. For RIA of sera, 0.1 ml of each serum was added to 0.2 ml of assay buffer. Then 0.1 ml of diluted antiserum (1:500 000) was added to each tube (standard curve and unknown samples) except those used for nonspecific binding and TC. Approximately 50 000 cpm of bovine ^{125}I -PAG in 0.1 ml buffer was added, immediately or after a 16-h preincubation at room temperature, to each assay tube. The final incubation volume was 0.5 ml. After incubation for 16 h at either 37°C or room temperature, the free and bound fractions were separated by addition of 1 ml (2.5 mg/ml) of sheep anti-rabbit IgG serum coupled to activated cellulose to each tube except TC and sample tubes. After a further 2-h incubation at room temperature, each tube was centrifuged at $1\,500 \times g$ for 10 min and the supernatant was discarded. The precipitate was washed with 3 ml of buffer and centrifuged again at $1\,500 \times g$ for 5 min, and the supernatant was discarded. The radioactivity of the precipitate was counted in an LKB (Rockville, MD) gamma counter (Model 1272 ClinGamma) with an efficiency for ^{125}I of 60%.

To determine accuracy of bPAG measurement in serum, known amounts of bPAG added to a pool of stallion serum (0.5, 1, 2, 4, 8, 15, 30, 62.5, 125, and 250 ng/ml) were assayed.

RESULTS

Assay Validation

The ^{125}I -radiolabeled bPAG (tracer) bound at a high antiserum final dilution of 1:1 500 000. That dilution bound 35–40% of ^{125}I -radiolabeled bPAG in the absence of unlabeled protein. In the presence of excess antibody, 90% of labeled bPAG was bound. This suggested an absence of contamination of the tracer preparation by immunologically inactive material [28]. To maximize sensitivity of the assay, the antiserum was used routinely at a final dilution of 1:2 500 000, which bound 20–25% of ^{125}I -radiolabeled bPAG with a nonspecific binding ranged from 98% to 1.16% binding when 0.02–10 ng of bPAG was added per assay tube. Sensitivity, defined as the lowest standard amount distinguishable from zero, was consistently 200.01 ± 4.10 pg/ml. Sensitivity of the RIA was higher when samples were incubated at room temperature for 16 h before addition of bovine ^{125}I -PAG and when a 2-h incubation period followed addition of tracer. The RIA was less sensitive when performed at 37°C (Fig. 1). Displacement of standard inhibition curves tested over 28 assays within a 3-mo period was highly repeatable and consistently ranged from $96.40 \pm 1.99\%$ to $3.31 \pm 0.93\%$ (means \pm SD) binding in buffer control tubes containing 0.1 ml of bPAG-negative serum when 0.02–5.00 ng bPAG was added.

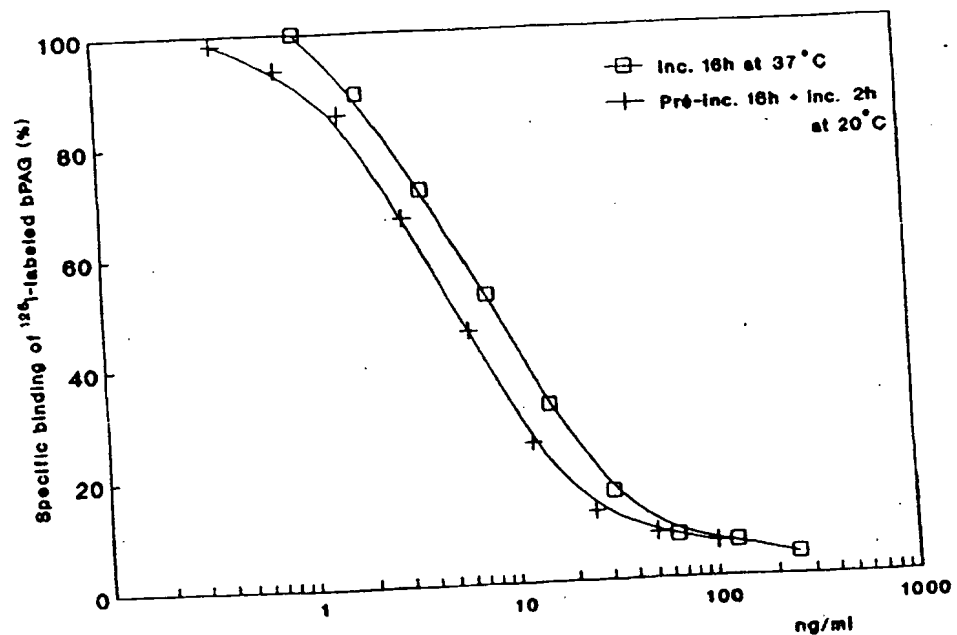


FIG. 1. Displacement curves in RIA for bPAG. Anti-bPAG antiserum at final dilution of 1:2 500 000 was incubated (Inc.) with ^{125}I -radiolabeled bPAG for 16 h at 37°C and for 2 h at $\pm 20^\circ\text{C}$ after 16 h of preincubation (Pre-inc) at $\pm 20^\circ\text{C}$.

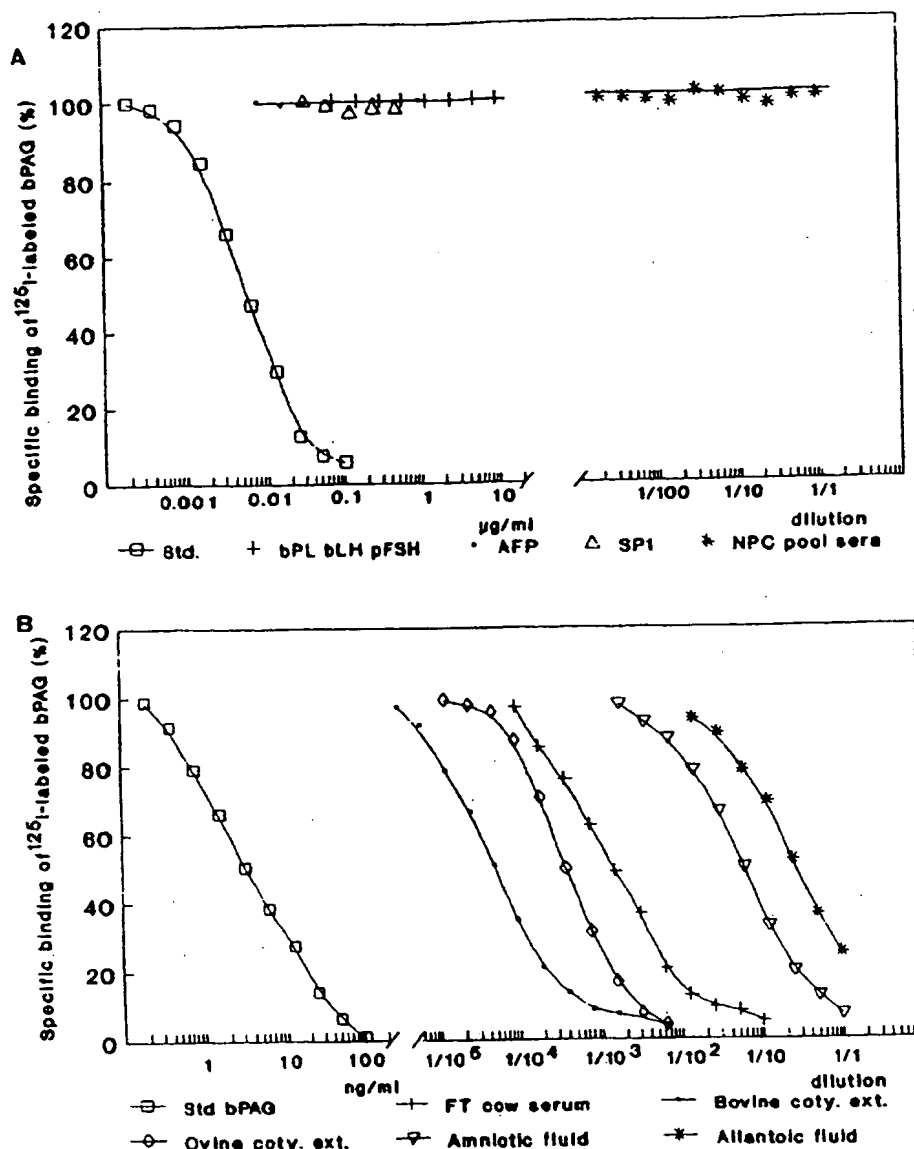


FIG. 2. RIA for bPAG. (A) No cross-reactivity was observed with bPL, bLH, pFSH (10 $\mu\text{g/ml}$), AFP (1 $\mu\text{g/ml}$), SP₁ (0.5 $\mu\text{g/ml}$), and pooled sera from nonpregnant cows (NPC pool sera) assayed at serial dilutions. (B) Cross-reactivity of full-term (FT) cow serum (1/10), bovine and ovine cotyledonary extracts (1/500), and fetal fluids (amniotic and allantoic) with antiserum (R498) to the mono P fraction of bPAG.

Sensitivity and specificity of the bPAG RIA are illustrated in Figure 2A. Gonadotropic hormones from pituitary (LH, FSH) and placental (eCG) origin, as well as placental (bPL, SP₁) and fetal proteins (AFP) from several species did not exhibit any inhibition of binding. Similarly, pooled sera from nonpregnant cows assayed in serial dilutions did not exhibit inhibition curves parallel to the bPAG standard curve. In contrast, full-term cow sera and bovine and ovine placental extracts and fetal fluids cross-reacted highly in the

assay. Except for ovine placental extracts, serial dilution of samples that cross-reacted in the bPAG RIA indicated that displacement curves were parallel to the bPAG standard curve (Fig. 2B).

Accuracy and mass recovery of the bPAG RIA were assessed in the range of 0.5–250 ng/ml and are presented in Table 1. The regression equation for the accuracy of bPAG recovered (Y) when known amounts of bPAG were added (x) to a pool of stallion sera was: $Y = 0.21 + 1.0x$, and the

TABLE 1. Recovery by RIA of bPAG added to 0.1 ml of bPAG-negative serum: total amount recovered (ng/ml) and percentage of recovery (%).

Amount added (ng/ml)	Total amount recovered* (ng/ml)	Recovery* (%)	Number of determinations
0.5	0.58 ± 0.07	117.35 ± 14.68	4
1	0.98 ± 0.09	99.69 ± 9.33	7
2	1.84 ± 0.08	96.16 ± 5.41	10
4	4.06 ± 0.18	103.83 ± 4.64	8
8	7.61 ± 0.22	97.39 ± 2.87	11
15	14.96 ± 0.32	96.28 ± 2.14	11
30	31.50 ± 0.58	100.80 ± 1.88	12
62.5	66.37 ± 1.87	104.60 ± 3.16	11
125	123.65 ± 1.81	99.08 ± 1.49	5
250	251.49 ± 7.73	100.59 ± 3.09	7

*Mean ± SEM.

correlation coefficient was $r = 0.99$. The intraassay (sera from 4 pregnant cows, 2 nonpregnant heifers, and 2 bulls assayed in duplicate and 10 times in each assay) and the interassay (sera from 3 pregnant cows, 1 nonpregnant cow, and 1 bull assayed in duplicate and in five consecutive assays) coefficients of variation were $6.87 \pm 2.48\%$ and $11.59 \pm 0.63\%$, respectively.

Measurement of Blood bPAG under Various Physiological Conditions

Of the 90 samples of fetal sera collected at different stages of gestation, bPAG concentrations ranged from 0.27 to 45.93 ng/ml in 82, and bPAG was undetectable (<0.2 ng/ml) in 8. In all cases, concentrations of bPAG in fetal sera were several orders of magnitude lower than those measured in the peripheral circulation of the dam. Concentrations of bPAG in fetal sera were highly variable; after adjustment for difference in stage of gestation (co-variable), bPAG concentrations from male and female fetuses did not differ ($p > 0.1$; 3.44 ± 0.48 vs. 2.60 ± 0.66 ng/ml, respectively). Bovine PAG concentrations in fetal sera (Y) were higher during the early (< 4 mo) stages of gestation (x) than during the later stages (> 4 mo) and are described by the following polynomial regression equation: $Y = 31.06 - 8.3x + 0.6x^2$; ($r^2 = 0.32$, $p < 0.08$; Fig. 3).

Maternal bPAG Concentrations during Gestation

Serum bPAG concentrations in pregnant cows bled daily from Days 20–35 pc and then at 2-wk intervals until Day 100 pp are presented in Figure 4. Serum bPAG concentrations were 0.38 ± 0.13 ng/ml (mean ± SD) on Day 22 pc and rose continuously as pregnancy advanced. After Day 240 pc, bPAG concentrations were greater than 158.90 ± 60.20 ng/ml, and they increased rapidly to 1551.90 ± 589.70 ng/ml at Day 270 pc. The increase became dramatic during the 10-day prepartum period and reached a peak concentration of 2462.40 ± 1017.90 ng/ml on Days 1–5 prepartum. After delivery, serum bPAG concentrations declined steadily to approximately 499.60 ± 267.20 ng/ml at Day 14

pp, 131.70 ± 77.90 ng/ml at Day 30 pp, and 10.10 ± 7.80 ng/ml at Day 60 pp. The undetectable level (<0.2 ng/ml) was reached only by Day 100 ± 20 pp.

Effects of type of recipient and sex of fetus on peripheral concentrations of bPAG in Holstein heifers, Holstein cows, or Hereford cows carrying purebred Holstein fetuses during the peripartum period are presented in Figure 5. Mean peripartum bPAG concentrations were higher in Hereford cows than in Holstein heifers or Holstein cows (3500 vs. 2300 and 1500 ng/ml; SEM = 400; $p < 0.003$). In addition, as illustrated in the bottom panel of Figure 5, Hereford cows carrying female fetuses had appreciably higher peripartum profiles of bPAG concentrations than those carrying male fetuses. No such differences due to sex of fetuses were detected in the two other types of recipient (breed of recipient × sex interaction, $p < 0.01$). Holstein recipients carrying male fetuses had higher prepartum profiles ($p < 0.05$) than those carrying female Holstein fetuses. Likewise the profile of peripartum increases in bPAG concentrations was of higher magnitude ($p < 0.01$) in Hereford cows than in Holstein heifers and in Holstein cows (Fig. 5, bottom panel). Peak values reached by Days 5 to 1 prepartum were 7600, 4200, and 3000 ng/ml in Hereford cows, Holstein heifers, and Holstein cows, respectively.

Pregnancy Diagnosis

Measurement of bPAG concentrations in sera collected from 430 Holstein-Friesian heifers (which had received transferred embryos) on Day 35 pe was used as pregnancy diagnosis. Pregnancy was confirmed by rectal examination performed at Day 45 pe. A total of 287 heifers were identified as pregnant and 143 heifers as nonpregnant according to bPAG concentrations measured by RIA (<0.5 ng/ml). Diagnosis of pregnancy by rectal palpation indicated that 267 heifers of the 287 were pregnant and that 140 of the 143 were nonpregnant. Not accounting for possible pregnancy loss between diagnosis by bPAG RIA and diagnosis by rectal palpation, false-positive results occurred in 6.90% (20/287) of the cases. False-negative results occurred in 2.10% (3/143) of the cases (Table 2). The total accuracy was 94.65% (407/430).

Possible Accessory Sources of bPAG

Detectable levels of bPAG or bPAG-like protein were measured in 7 of 30 unbred heifers (mean ± SD; 0.30 ± 0.09 to 0.50 ± 0.17 ng/ml) and in 3 of 20 bulls (3.01 ± 1.73 to 4.75 ± 1.42 ng/ml) selected at random. Detectable bPAG concentrations in sera from nonpregnant heifers determined the threshold level of >0.5 ng/ml that was adopted for positive pregnancy diagnosis.

DISCUSSION

This paper is the first to describe a highly specific and sensitive RIA for a well-characterized 67 000 *M*, pregnan-

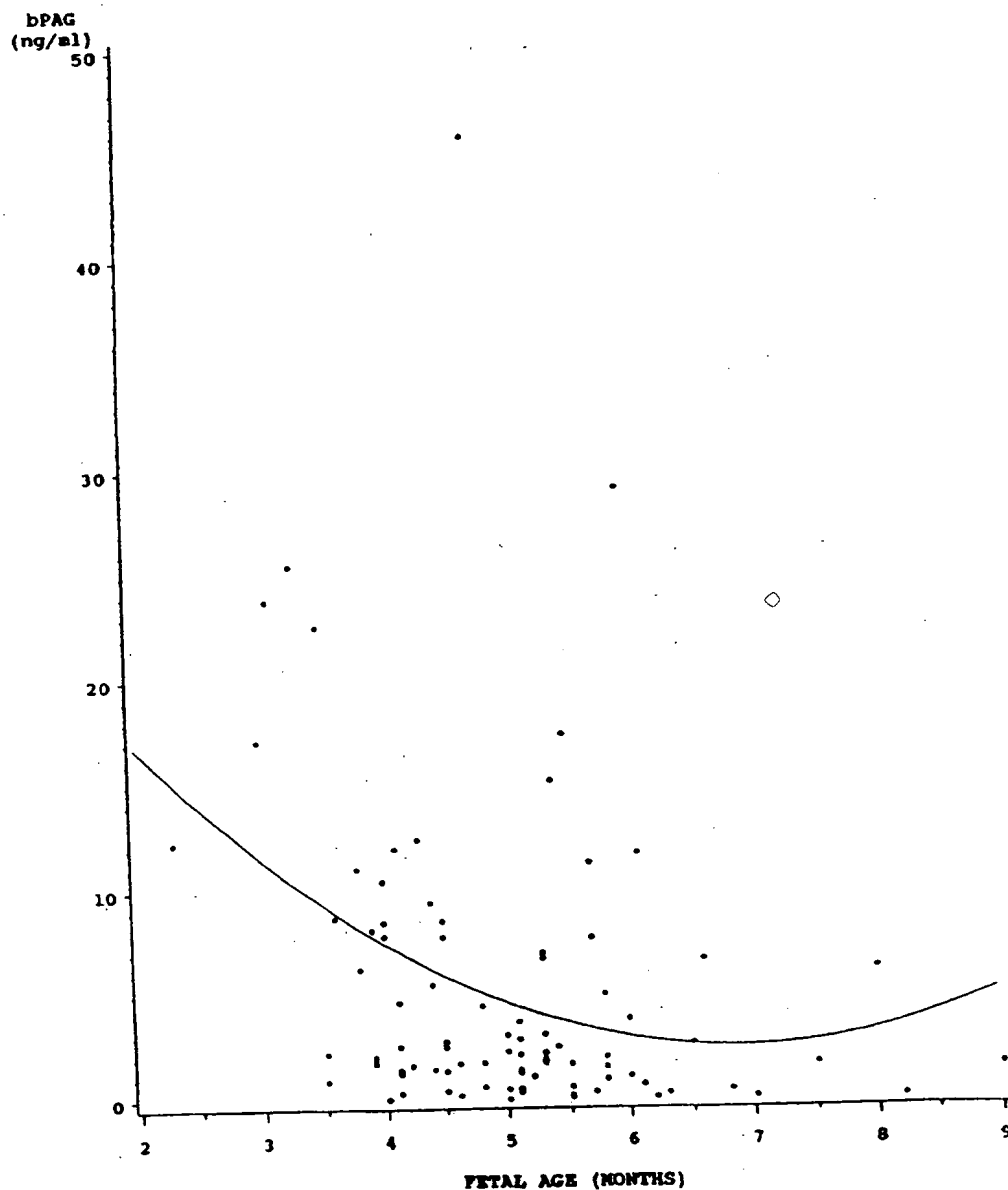


FIG. 3. Relationship between serum bPAG concentrations (Y) and fetal age (x). The regression equation is $Y = 31.06 - 8.3x + 0.6x^2$ ($r = 0.32$, $p < 0.08$).

associated glycoprotein in the bovine species [18,29]. The parallel inhibition binding curves observed with serial dilutions of placental extracts, pregnant cow sera, fetal sera, and amniotic and allantoic fluids suggest that the glycoprotein isolated and purified from bovine fetal cotyledons [18] is the same as that found in the placenta, in fetal and maternal sera, and in fetal fluids. Considering the higher concentration of bPAG in maternal serum than in fetal serum, the glycoprotein synthesized by the fetal placenta is se-

creted essentially into the maternal circulation. The lack of inhibition of binding observed with sera pooled from non-pregnant cows suggests that the bPAG is specific to, or at least highly associated with, pregnancy. However the nearly parallel inhibition binding curve of ovine placental extracts (Fig. 2B) suggests that bPAG and its ovine analog have high immunological homology.

Although the human SP₁ is also of trophoblastic [30] origin and is pregnancy-specific, no inhibition of binding was

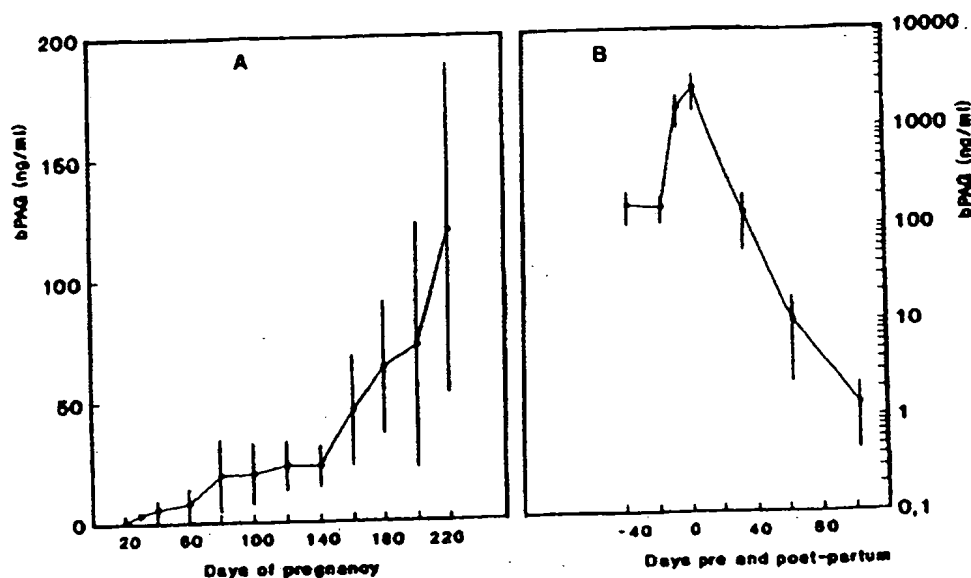


FIG. 4. Profile of the concentrations (mean \pm SD) of bPAG in serum of 20 cows bled from Day 20 of pregnancy until 100 days after parturition. Note the linear (A) and the logarithmic (B) scales of the Y axis respectively from 20 to 220 days of gestation and from Day -40 prepartum to Day 80 pp.

observed in RIA (Fig. 2A). Similarly, pituitary and placental gonadotropic hormones from several species (bPL, bLH, pFSH, and eCG) and fetal or serum proteins (AFP and BSA) at doses up to 10 μ g/ml were not detectable. In this regard, the present RIA results differ from those of Sasser et al. [17], who observed that bLH and ovine FSH inhibit the binding of 125 I-PSPB in a manner parallel to the standard curve. It is difficult to know whether PSPB and bPAG are quite different proteins or belong to the same family without comparing the amino acid sequences or immunologically testing the two glycoproteins.

The selective secretion of bPAG into the maternal circulation suggests that it might act mainly on the maternal side. A negative correlation was clearly established between fetal bPAG concentrations and fetal age (Fig. 3).

Up to Day 80 of pregnancy, all cows studied seemed to have similar concentrations of serum bPAG. After Day 80 of pregnancy, differences appeared among animals. The bPAG levels were consistently higher than those reported by Sasser et al. [17] for PSPB. The profile observed in the present study is quite different from those of bPL [31] and SP₁ [32]. The high increase of bPAG within the last 10 days prior to calving might be due to the possible involvement of the protein in the induction of parturition.

After delivery, bPAG concentrations decreased steadily, but not as rapidly as SP₁, which disappears from maternal serum about 2 wk after parturition [32], or bPL and oPL [33, 34]. The long time needed for bPAG to be cleared from the maternal serum might be the result of the very high concentration reached around parturition. It might also

result from the probable long half-life of the protein in blood, as observed by other workers for placental proteins like PSPB and eCG [35, 36]. Overall, the presence of bPAG in sera for nearly 100 days pp constitutes a problem for subsequent diagnosis of pregnancy by this method if re-breeding occurs less than 80 days pp. Another question is whether the remaining bPAG plays any role in postpartum anestrus.

Results of peripartum investigations suggest that peripheral concentrations of bPAG, as demonstrated with other placental proteins or hormones [33, 37], are affected by the maternal environment but are also controlled by fetal genotype (sex and family of fetus). The explanation of such a phenomenon might be found in the fact that trophoblast cells express some types of antigens that are recognized as foreign by the mother [37]. If so, it is reasonable to assume that similar antigens will be expressed to some degree by the trophoblast of crossbred conceptuses but to a greater extent by the trophoblast of fetuses of breeds unrelated to that of the recipient. As shown by Allen for eCG in horses and donkeys [37], the "cell-mediated reaction" is also the probable explanation of higher bPAG prepartum concentrations in Holstein heifers carrying purebred Holstein fetuses. Indeed, it has been demonstrated elsewhere that females in their first pregnancy produce high titers of antibodies against some trophoblastic antigens recognized as foreign to the maternal immune system [37]. Given the immunosuppressive properties of bovine [38] and human [39] pregnancy-specific or pregnancy-associated proteins and given the possibility that bPAG and PSPB belong to the same

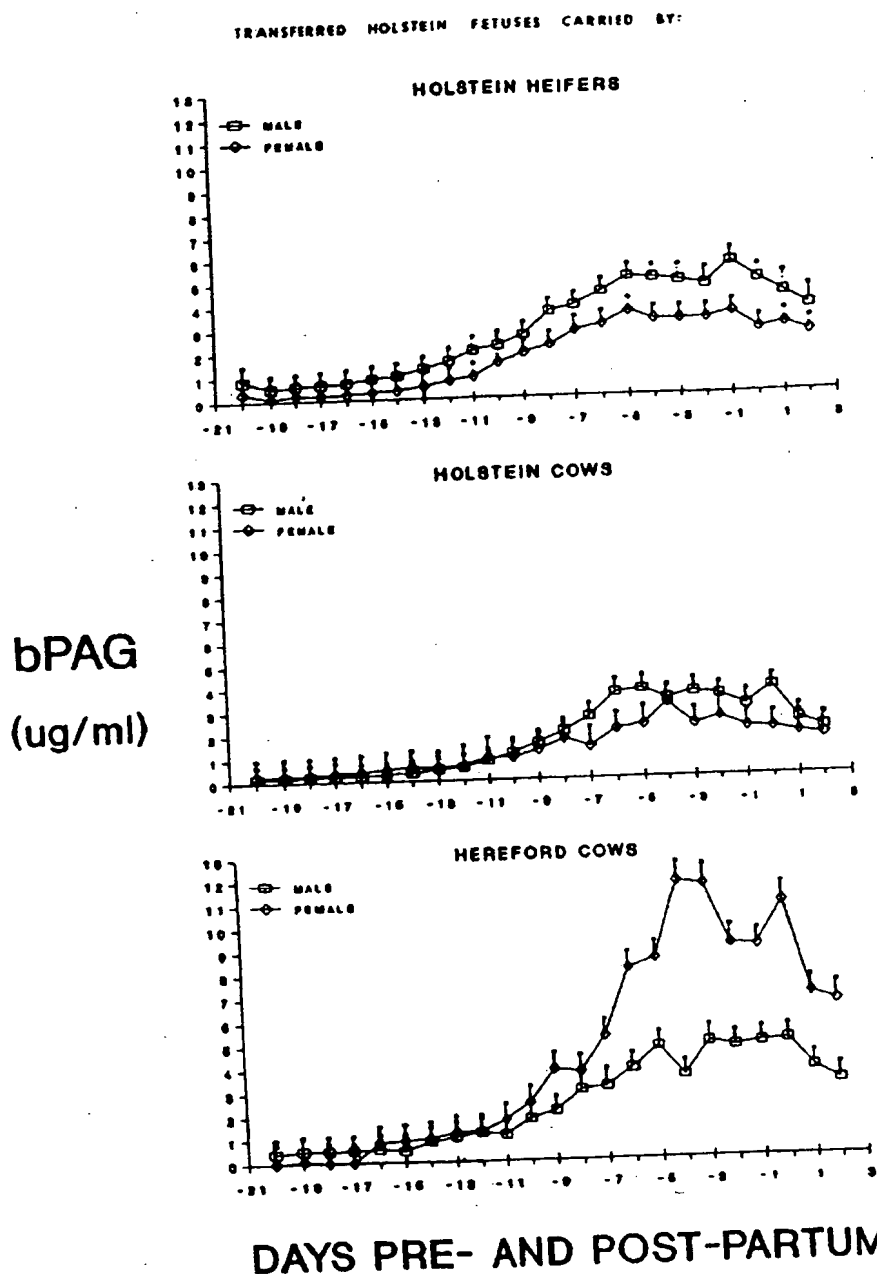


FIG. 5. Peripartum bPAG concentrations in Holstein heifers (top panel), Holstein cows (middle panel), and Hereford cows (bottom panel) carrying purebred Holstein male or female fetuses.

family, it may be postulated that bPAG constitutes a protective mechanism that either masks the trophoblast antigens from maternal immune recognition or suppresses or neutralizes any maternal reaction that would otherwise be stimulated [37] in later pregnancies. Alternatively, differences in peripartum bPAG concentrations that are related

to the type of recipient or sex of fetus may also reflect differences in placental function. As observed for bPAG in the present experiment, peripartum concentrations of estrone sulfate, which is mostly of placental origin, are increased when fetal and maternal units are genetically different [40]. It has been reported that greater antigenic dissimilarity is

TABLE 2. Detection of pregnancy by RIA for bPAG in serum (Day 35 pe) and by rectal palpation (Day 45 pe) in 430 Holstein-Friesian heifers that had received a transferred embryo.

Method of Detection			
RIA	Rectal Palpation		Total
	+	-	
+	287 (93.03%)*	20 (6.87%)	287
-	3 (2.10%)	140 (97.90%)	143
Total	270	160	430

*Numbers in parentheses indicate accuracy; total accuracy was 94.65%.

related to larger placentas in mice [41] and higher calf birth-weight in cows [42].

The accuracy of pregnancy diagnosis using milk progesterone concentrations at Day 24 after breeding and ultrasonography from Days 21 to 35 is high [43, 44]. The bPAG, a glycoprotein of placental origin, could be considered as a marker of placental function. It is detectable in the maternal serum throughout gestation until Day 100 \pm 20 pp. The sensitive, specific, and accurate RIA of bPAG could be used as a serological method for early pregnancy diagnosis in cattle. With the bPAG assay, the date of mating or artificial insemination is not needed for pregnancy diagnosis as it is for progesterone assay. In the present study, the accuracies of the test for pregnancy and nonpregnancy were 93.03% and 97.90%, respectively. The overall accuracy of the test was 94.65%. The high rate of false-positives might be due, in part, to embryonic deaths that occurred a few days before blood sample collection or between Days 35 and 45 pe. The false-negative results were probably the consequence of very low serum bPAG concentrations at the time of blood sample collection due to among-animal variations in bPAG production.

The assay results in some bulls and unbred heifers were surprising unless one accepts the existence of accessory sources of bPAG. In humans, extraplacental production of so-called trophoblast-specific proteins (e.g., SP₁) by nontrophoblastic cells is a well-recognized phenomenon [45, 46]. Bovine PAG or a bPAG-like protein has been found in bull and ram sera and testes [47]. This suggests that bPAG may also exist in extraplacental sources. Given this possibility, the apparent detection of very low concentrations of bPAG may not necessarily indicate pregnancy. The bPAG concentration at or above which a diagnosis of pregnancy can be confidently made should be determined so that assay background or nonspecific "noise" levels (0.5 ng/ml) can be accounted for. For this reason, results of any bPAG assay before Day 28 pc should be interpreted cautiously.

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Characteristics of pregnancy-specific protein B in cattle

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Summary. Pregnancy-specific protein B (PSPB) has been isolated from placental tissue of cows. Antisera were developed against PSPB and by immunohistochemical techniques the protein was localized to the binucleated cells of the trophoblastic ectoderm. A radioimmunoassay (RIA) was also developed and used to detect PSPB in sera of pregnant animals. The RIA has been used successfully in pregnancy testing in cattle and other ruminants. The assay can also be used to detect time of embryonic death. Chemical characterization of PSPB showed that it was an acidic glycoprotein with an apparent molecular weight of 78 000. It has several isoelectric variants with pIs of 4.0-4.4.

Keywords: pregnancy; protein; pregnancy-specific protein B; embryo; cattle

Introduction

Proteins produced by the placenta have been reported for many years in several species. For example Aschheim & Zondek (1928) reported that a protein named human chorionic gonadotrophin (hCG) was present in urine of pregnant women. This can be measured in urine or blood at about 10 days after conception (Marshall *et al.*, 1968). Several other pregnancy proteins have been found in the human but not all reach the maternal blood or urine (Bohn, 1985).

Several pregnancy proteins are reported in domestic animals. Pregnant mare serum gonadotrophin was found in serum of mares (Cole & Hart, 1930) and used as a pregnancy marker (Cole & Hart, 1942). Rowson & Moor (1967) showed that a heat-labile substance in the 13-day-old sheep embryo, when infused into the uterus of the cyclic sheep, would extend the life-span of the corpus luteum. Later, Godkin *et al.* (1982) studied proteins produced by the conceptus of this species and showed that the major secretory protein was a low molecular weight acidic protein produced between Days 13 and 21 of pregnancy. This was termed ovine trophoblast protein-1 (oTP-1). This protein is probably the same as trophoblastin, a substance earlier identified by Martal *et al.* (1979). oTP-1 is secreted at a time corresponding to the time of maternal recognition of pregnancy in the ewe and is involved in regulation of protein secretion by the endometrium (Vallet *et al.*, 1987). Chemical characteristics of this protein suggest that it is similar to a human alpha interferon (Imakawa *et al.*, 1987). As in sheep, the early bovine conceptus, when homogenized and infused into the uterus, will extend luteal life-span (Betteridge *et al.*, 1980; Northey & French, 1980; Dalla Porta & Humblot, 1983). The major protein secreted by the bovine embryo between Days 16 and 24-27 has properties similar to those of oTP-1 (Bartol *et al.*, 1984) and is termed bovine trophoblast protein-1. Godkin *et al.* (1988) have identified several proteins of the bovine conceptus near Days 30 to 40 of gestation.

Pregnancy-specific protein B (PSPB) has been found in the serum of pregnant cattle (Sasser *et al.*, 1986) and has been used as a pregnancy marker. This paper will summarize currently understood biological and chemical characteristics of this protein.

Pregnancy-specific protein B

This protein was found after immunizing rabbits with homogenates of whole bovine placenta and adsorbing the antisera with somatic tissues to remove antibodies to proteins not specific to the placenta. Remaining antibodies were against alpha-fetoprotein and PSPB of the placenta (Butler *et al.*, 1982). Adsorbed antisera were used as a marker in immunodiffusion and immunoelectrophoresis methods to isolate a preparation of PSPB (R-37) from bovine placenta (Sasser *et al.*, 1986). A new antiserum (RGS 38-1) was then developed in a rabbit for use in development of a double-antibody radioimmunoassay (RIA) for PSPB (Sasser *et al.*, 1986) and for further characterization of PSPB.

Biological characteristics

Source of PSPB. Immunohistochemical methods were used to find that PSPB was present in the giant binucleated cells of the trophoblast of the placenta (Eckblad *et al.*, 1985). Also, studies by Reimers *et al.* (1985) showed that PSPB secretion into media was greater by placental cell fractions enriched in binucleated cells than by those enriched in mononucleated cells.

The presence of PSPB in body fluids other than blood has been investigated. Cross-reacting antigens have not been found in urine, tears, saliva, or vaginal or cervical secretions; however, milk contains PSPB when collected at a time when it is excessively high in plasma of cows, i.e. within the week after parturition (R. G. Sasser, unpublished).

Pregnancy testing with PSPB. Cross-reactions of antigens in sera from the cow, sheep and goat, compared to the PSPB standard for this RIA, are shown in Fig. 1. The sera of pregnant cows paralleled the standard curve while that of pregnant sheep and goats did not. The assay was therefore quantitative for cow but only qualitative for sheep or goat PSPB. However, a qualitative assay is adequate for a test for pregnancy. There were no cross-reacting antigens in sera of non-pregnant animals and so the RIA can be used to detect pregnancy in these ruminants.

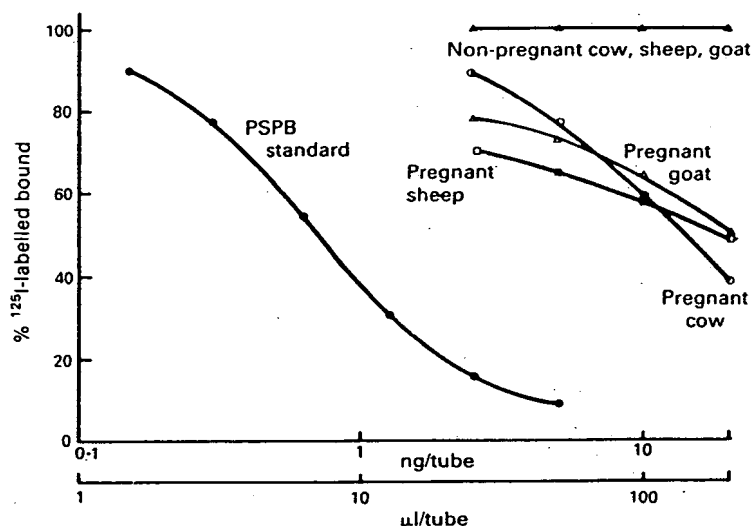


Fig. 1. Cross-reaction of antigens in sera of pregnant sheep, goats and cows with Antiserum RGS 38-1 compared to R-37 PSPB standard. (From Sasser & Ruder, 1987.)

Sasser *et al.* (1986) have shown that the RIA for cattle was appropriate for pregnancy testing in large numbers of animals. Additionally, they showed that PSPB was present in serum from the 3rd week until the end of pregnancy in most cows and, in some, as early as the 2nd week of gestation.

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The time during the 3rd–4th week that the test could first be used and detect a high percentage of pregnancies was evaluated in dairy cattle by Humblot *et al.* (1988a). Cows (N = 76) and heifers (N = 71) were subjected to 177 artificial inseminations (AI) followed by analyses of blood for PSPB by RIA on Days 24, 26 and 30–35 and some on Day 70 after AI. All data were compared for accuracy with rectal examination data at Day 70.

As shown in Table 1, accuracy improved as gestation age increased. The accuracy of the test was acceptable by 30–35 days and was considerably more accurate than the milk progesterone assay administered to these same animals on Days 22 and 24 after insemination: 70, 77 and 79% on Days 22, 24, and Days 22 + 24 combined.

Table 1. Accuracy of pregnancy detection at different days of pregnancy in 177 cows and heifers

Pregnancy status at 70 days	Day of gestation			
	24	26	30–35	70
Pregnant	86 (50/58)	88	90 (83/92)	99 (77/78)
Non-pregnant	72 (86/119)	89 (84/94)	100 (83/83)	100 (36/36)
Overall	77 (136/177)	89 (157/177)	95 (166/175)*	99 (113/114)†

Values are % with the numerator being the total no. of pregnant or non-pregnant animals by PSPB assay and the denominator the no. that returned to oestrus before 70 days or were palpated at 70 days as pregnant or non-pregnant.

*Two animals returned to oestrus and were not included in this calculation.

†Serum was collected and analysed for PSPB on Day 70 in only 114 of the 177 animals initially started on the project.

The sensitivity of the RIA has been modified by changing first antibody dilution and incubation conditions. Estimates of pregnancy with high accuracy before 30–35 days are anticipated (R. G. Sasser, unpublished).

Post-partum PSPB. Ruder & Sasser (1986) showed that PSPB remains in the serum for a considerable time after parturition in cows. The half-life was estimated to be 7.3 days from Days 21 to 53 *post partum* in cows which were hysterectomized on Day 21. This long half-life poses a problem for pregnancy testing in re-mated post-partum cows. Studies are being conducted with the above-mentioned sensitive RIA to determine the time that an acceptable low level of PSPB is reached during the post-partum interval in order that a higher level of PSPB of a new pregnancy at 26–28 days can be determined.

Estimates of embryo death. We have also shown that PSPB can be used in conjunction with the milk progesterone assay for monitoring time of embryonic death (Humblot *et al.*, 1988b). Milk or serum progesterone concentration at 21–24 days is indicative of the presence or absence of an embryo at 16–17 days since this is the time that the embryo signals its presence to the maternal system (Northey & French, 1980). Later embryo death can be determined by the PSPB assay. The time of embryo death can therefore be surveyed from Day 16 until term pregnancy.

Chemical characteristics

Since the success of the RIA for pregnancy detection relies upon the displacement of radio-labelled PSPB (preparation R-37), all chemical isolation and analysis has relied upon displacement of radiolabelled PSPB from Antiserum RGS 38-1. Additionally, polyacrylamide gel electrophoresis (PAGE) has been analysed by Western blotting of immunoreactive proteins on nitrocellulose paper using Antiserum RGS 38-1 (R. G. Sasser & J. Crock, unpublished).

The R-37 preparation of PSPB contained several immunoreactive bands as determined by Western blotting after SDS-PAGE. Most were removed by column chromatography of radio-labelled PSPB during the iodination procedure (Sasser *et al.*, 1986) and before use in the RIA. A band containing the majority of the radioactivity remained and had an apparent molecular weight of 78 000 (SDA-PAGE estimate).

Extraction of PSPB from bovine cotyledons yielded a single major immunoreactive band and 2 minor bands with apparent respective molecular weights of 78 000, 85 000 and 90 000 by SDS-PAGE. Migration of the band of M_r 78 000 was not affected by β -mercaptoethanol, suggesting that PSPB exists as a monomer and that disulphide linkages are not important in maintaining tertiary structure of the molecule.

Cultures of whole cotyledons from bovine placenta of 90 days of age secreted proteins into medium (Hanks' balanced salt solution). Five proteins, when separated on SDS-PAGE were detectable by Antiserum RGS 38-1 on a Western blot. The major band had an estimated molecular weight of 78 000 and 4 minor bands had molecular weights of 48 000, 65 000, 85 000 and 90 000. Antisera were produced against the bands of M_r 78 000, 85 000 and 90 000 which had been cut from PAGE gels and injected, with polyacrylamide, into rabbits. Immune sera against each antigen cross-reacted with the other two in the Western blot. All antisera also bound the radiolabelled R-37 preparation of PSPB.

Radiolabelled R-37 preparation of PSPB and the M_r 78 000 preparation from culture media were subjected to two-dimensional IEF-SDS-PAGE. Both of these proteins of M_r 78 000 contained 7 isoelectric variants with isoelectric points between 4.0 and 4.4. Two variants with pIs of approximately 4.2–4.25 were most abundant. Peptide maps of these isomers were similar, indicating that all variants are charge isomers and not different proteins with similar molecular weights and pIs. The protein of M_r 78 000 contained ~5% hexose sugars and ~3% sialic acid.

Conclusion

PSPB is a glycoprotein produced by the binucleate cells of the placenta of ruminant animals. The biological function of this protein awaits determination. Measurement of it in plasma or serum but probably not in other biological fluids can be used as a pregnancy test. Care must be taken in evaluating sera of animals in the early post-partum period for a new pregnancy. A false positive test may be obtained.

It is anticipated that PSPB can be formatted into a field test kit that can be purchased by producers and applied at their own facilities. Such tests are available for measurement of other chemical substances. Actual assay time is only 3–10 min.

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Multiple Pregnancy-Associated Glycoproteins Are Secreted by Day 100 Ovine Placental Tissue¹

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ABSTRACT

Pregnancy-associated glycoprotein (PAG)-1 (PAG1) and pregnancy-specific protein B are either identical or closely related antigens released by trophoblast binucleate cells of placentas of cattle. Sheep and other ruminants produce similar products. There is evidence, however, that these antigens, which are related structurally to the pepsinogens and other aspartic proteinases, are not single gene products but members of an extensive family. Here, the sequential use of ammonium sulfate precipitation and Sepharose blue, anion-exchange, and cation-exchange chromatographies, as well as isoelectric elution from a Mono P column, has allowed several PAG1-related molecules to be purified from the medium after culture of explants from Day 100 sheep placentas. Each of these PAGs cross-reacted to a varying extent with a panel of three different anti-PAG1 antisera. Four of them, all of which were major secretory products of the placenta, were subjected to amino-terminal microsequencing. Although each was related to ovine (ov) PAG1, none was identical. Reverse transcription-polymerase chain reaction was then used to amplify PAG1-related cDNA from Day 100 placental RNA. Seven novel full-length cDNA, all distinct from ovPAG1, were identified from 25 cDNA selected for sequencing. Only two of these (ovPAG3 and ovPAG7) encoded polypeptides identical in sequence at their inferred amino termini to one of the PAGs (ovPAG₆₅) purified from explant cultures. Even so, they were only 84% identical in overall sequence. The remaining five cDNA were unique. In situ hybridization analysis revealed that expression of ovPAG3 and ovPAG7, like that of ovPAG1, is confined to trophoblast binucleate cells. The data confirm that at Day 100 of pregnancy the ovine placenta produces many different PAGs, which differ considerably in sequence and immunological cross-reactivity.

INTRODUCTION

Pregnancy-associated glycoprotein (PAG)-1 (PAG1) [1], also known as pregnancy-specific protein B (PSPB) [2], was identified by two different groups of investigators who used essentially comparable approaches in their experiments. Both obtained an initial antiserum by injecting rabbits with a crude mixture of bovine placental proteins and by adsorbing out nonrelevant antibodies on tissue extracts and red blood cells from nonpregnant cows. The resulting preparations were used to monitor the purification of the pregnancy-associated antigens themselves from placental extracts, which in turn were employed to generate more specific "second-generation" antisera useful for detection

of pregnancy in cattle [3–6]. In the case of the pioneering studies of Sasser and colleagues, it has become clear that the PSPB used to immunize the rabbits was only partially purified [2]. In addition, a series of molecular weights have been published, ranging from ~50 000 [2] to 78 000 [3], and Western blotting has been reported to show several bands of immunoreactive protein [5]. Independently, Mialon et al. [7, 8] described a 60-kDa placental protein that was also pregnancy specific and presumed to be distinct from PSPB. The PAG1 antigen purified by Zoli et al. [1] had a molecular weight of 67 000 and appeared pure as judged by Edman sequencing. Nevertheless, it was composed of several isoelectric variants believed to be the result of a varying content of sialic acid. Moreover, the amino-terminal sequence obtained was similar, but not identical, to that inferred from the bovine (bo) PAG1 cDNA described below.

A cDNA for boPAG1 was cloned [9] by screening a placental library with the antiserum prepared by Zoli et al. [1], but the same procedure also identified a less common cDNA encoding a related polypeptide too distinct in sequence to be an allele of PAG1 [10]. At about the same time, an antiserum raised against yet another placental protein identified a third bovine PAG [11], and Southern genomic blotting strongly suggested that there were multiple PAG genes in cattle [10]. Most surprising was the discovery that the PAGs were members of the aspartic proteinase gene family and were closely related in structure to the pepsins [9–11]. Despite this similarity to a proteolytic enzyme, mutations within or near the active sites of bovine PAG would likely have rendered the bovine PAG catalytically inactive [9, 12].

PAG-related antigens have been reported in several ruminant ungulate species other than cattle [13–17], and PAG cDNA have been cloned from both the sheep [9, 18], a ruminant, and the pig [19], a monogastric species. In both the latter species, at least two distinct cDNA were identified.

Together, the results outlined above suggest that the PAGs may exist as a more extensive family than originally suspected and that the various antisera that have been generated have not necessarily been equivalent in cross-reactivity. Such suspicions have been strengthened by the observation of Atkinson et al. [20], who examined the specificity of the monoclonal antibody SBU-3, which had been selected on the basis of its ability to stain ovine trophoblast binucleate cells. This antibody recognizes a carbohydrate epitope of as yet unknown structure but was also able to form immune complexes with several different proteins in placental extracts that had amino-terminal sequences similar to that of ovine (ov) PAG1. OvPAG1 was not itself immunoprecipitated.

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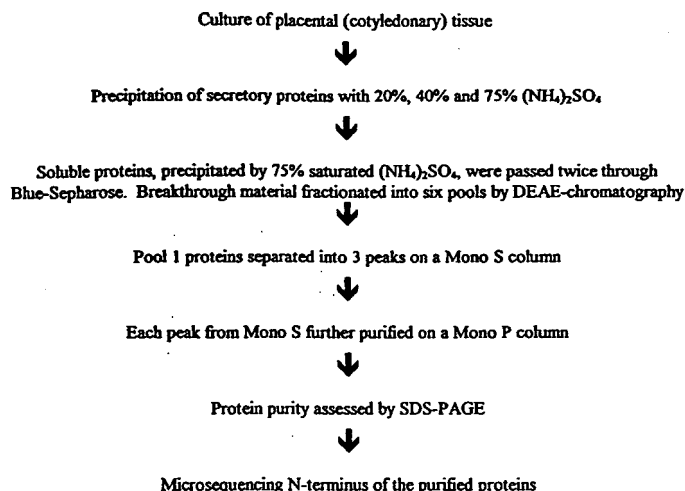


FIG. 1. A schematic outline of ovPAG purification. Secretory proteins from Day 100 ovine placenta-conditioned medium were first precipitated with ammonium sulfate. The material collected in the 40–70% ammonium sulfate fraction was dialyzed in 10 mM Tris (pH 8.0), concentrated, and further analyzed by DEAE-Sepharose chromatography. The fractions containing materials cross-reactive to anti-PAG antisera were pooled and further separated on Mono S and Mono P HPLC columns. The purified ovPAGs were sequenced at their amino termini.

The present experiments were conducted to verify that multiple kinds of PAGs are secreted by the sheep placenta. They were also designed to provide an estimate of the amounts of PAG protein secreted, since the frequency of PAG transcripts in cDNA libraries, including those from the pig [19], seemed unusually high [9, 11, 21]. Finally, it was of interest to know whether the complement of PAGs produced at Day 100 of the 145-day pregnancy were among those already identified in the preimplantation period [18], in midpregnancy [20], and closer to term [9].

MATERIALS AND METHODS

Culture of Placental Tissue

Uteri were removed surgically from three Day 100 pregnant mixed-breed ewes and dissected open to expose the placenta in a tissue culture hood. Cotyledons were removed from the placenta as cleanly as possible and minced into small pieces (~5 mm³). These explants were washed three times in Dulbecco's Modified Eagle's medium (DMEM) containing penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone (0.5 µg/ml) and cultured in the same medium at 37°C under 5% CO₂:95% air for 8 h. A total of 852 g wet tissue was cultured in a total of 5 L of DMEM. The tissue and medium were separated by centrifugation after 8 h of incubation.

Purification of ovPAG from Cotyledon-Conditioned Medium

The purification of ovPAG is shown schematically in Figure 1. All procedures were carried out at 4°C except high-performance chromatography, which was performed rapidly at room temperature. Between purification steps, samples were stored at -20°C in buffers containing a cocktail of proteinase inhibitors (0.5 mM EDTA, 0.5 mM PMSE, and 0.4 µg/ml leupeptin). Before the beginning of each chromatographic procedure, all samples were dialyzed against the start buffer used to equilibrate the column and concentrated in Centrprep-30 cartridges (cutoff 30 kDa;

Amicon, Beverly, MA). Protein concentrations were determined by the Bradford procedure [22].

(NH₄)₂SO₄ precipitation. Proteins from 4 L of the conditioned medium were precipitated sequentially by addition of (NH₄)₂SO₄ to 20%, 40%, and 75% saturation. The pelleted proteins were resuspended and dialyzed against 10 mM Tris-HCl, pH 8.0. The majority of the protein (11.48 g) was precipitated between 40% and 75% saturated (NH₄)₂SO₄, while smaller amounts were recovered at 20% (290 mg) and 40% (5.8 g) (NH₄)₂SO₄. OvPAGs detected by immunoblotting and ELISA were present predominantly in the 40% to 75% (NH₄)₂SO₄ fraction.

Blue Sepharose chromatography. A portion (405 mg) of the protein that had been precipitated at 75% (NH₄)₂SO₄ saturation was applied to a Blue Sepharose column (20 × 1.3 cm; Sigma Chemical Co., St. Louis, MO) that had been equilibrated with 0.1 M sodium acetate, pH 7.0. This procedure was employed in order to remove much of the albumin present in the culture medium. The unabsorbed, breakthrough fractions (243 mg) contained all of the immunoreactive materials and were pooled for further purification of PAG. Proteins bound to the Blue Sepharose were eluted with a buffer containing 0.5 M NaCl in 0.10 M sodium acetate, pH 4.0.

Anion-exchange chromatography. The unabsorbed material from the Blue Sepharose column was loaded onto an open DEAE-Sepharose column (2.5 × 92 cm; Pharmacia, Kalamazoo, MI) that had been equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The column was washed extensively with the same buffer until the 280-nm absorbency reached baseline. Proteins were eluted from the column by a combination of a gradient of NaCl in 10 mM Tris, pH 8.0. Step 1 consisted of elution with a 1-liter linear gradient (1 mM to 100 mM NaCl). This was followed by washing of the column with three bed volumes of 100 mM NaCl. Step 2 was a 1-liter linear gradient (100–200 mM NaCl) followed by a wash with three bed volumes of 200 mM NaCl. In the final step, a third linear gradient (200–300 mM NaCl) was used. The column was then washed with 300 mM NaCl as described above. The eluate was collected in 9.5-ml fractions, monitored for absorbance at 280 nm, and assayed for the presence of PAG by ELISA. Immunopositive fractions were combined into 5 pools according to their absorbance at 280 nm and immunoreactivity. Only the analyses of pool 1 (D1) and pool 3 (D3) (see Figs. 2 and 3) are reported here.

Cation-exchange HPLC. The DEAE pools were separately analyzed by chromatography on a Mono S HR5/5 column (Pharmacia). The column was washed with 20 mM sodium acetate (pH 5) at a flow rate of 1 ml/min for 30 min. The proteins were then eluted by using a linear gradient (0–1 M NaCl in 20 mM sodium acetate, pH 5) at a flow rate of 1 ml/min. The fractions (1 ml/tube) were collected and assayed for ovPAG by using an ELISA or Western blotting.

Chromatofocusing HPLC. Immunoreactive pools from the Mono S column were subjected to isoelectrofocusing on a Mono P HR5/20 column (Pharmacia). The column was equilibrated with start buffer (0.025 M bis-Tris, pH 6.3). Proteins were eluted with polybuffer 74, pH 4.0 (Pharmacia), at a flow rate of 0.5 ml/min. Each protein peak was analyzed by Western blotting after proteins were separated by SDS-PAGE.

N-Terminal Amino Acid Sequence Analysis

The purified ovPAG was transferred to polyvinylidene difluoride (PVDF) membranes with a ProSpin device (Ap-

plied Biosystems, Foster City, CA) and sequenced by the Edman degradation method on an Applied Biosystems Model 470 protein sequencer with online analysis for phenylthiohydantoin derivatives (Protein Core, University of Missouri-Columbia, Columbia, MO). One fraction (see lane 14 in Fig. 7) contained two separable immunoreactive bands. Proteins in this fraction were separated by SDS-PAGE, stained with Coomassie blue, and then transferred to a PVDF membrane for sequencing.

Anti-PAG Antisera

The preparation of anti-native ovPAG1 [9, 23] and anti-native boPAG1 antisera [1, 6] has been described previously. The anti-recombinant ovPAG1 serum was raised in rabbits against ovPAG1, which had been produced in *Escherichia coli* strain BL21 (DE3)pLysS as previously described [24]. Briefly, the coding region of ovPAG1 (without the signal peptide) was amplified by polymerase chain reaction (PCR) and engineered to contain in-frame, flanking *ndel* restriction sites for cloning into the pET11a vector (Invitrogen, San Diego, CA). Recombinant ovPAG1 expression was induced with 1 mM isopropyl β -D-thiogalactopyranoside. The protein was produced as insoluble inclusion bodies that were isolated by centrifugation from French-pressed cells. The inclusions were washed two times in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 20 μ M PMSF, and 1% Triton X-100 and solubilized in 8 M urea, 20 mM Tris-HCl (pH 8.0), 100 mM 2-mercaptoethanol, and 1 mM EDTA. The turbid solution was then cleared by centrifugation. The solubilized proteins (250 μ g) were mixed with 0.5 ml Freund's complete adjuvant (Gibco, Chagrin Falls, OH) and injected s.c. into two New Zealand white rabbits [25]. The rabbits were reimmunized at 5-wk intervals with 100 μ g of the recombinant protein in Freund's incomplete adjuvant. Antiserum was collected from the central ear vein 2 wk after each immunization [25].

Detection of ovPAGs by Western Blotting and on Dot Blots

To denature proteins before electrophoresis, the samples were boiled for 5 min in loading buffer that contained 1% SDS and 1% 2-mercaptoethanol. The denatured proteins were separated in a 10% polyacrylamide gel containing 0.1% SDS and then either stained with Coomassie blue (loading: 10 μ g/lane) or transferred to a nitrocellulose membrane by electroblotting (loading: either 2 or 0.5 μ g/lane). For dot blotting, proteins (1 μ g) were directly spotted onto nitrocellulose membranes. Both kinds of blots were then exposed to 5% (w/v) milk proteins, washed, and reacted with either preimmune serum, anti-native ovPAG1, anti-native boPAG1, or anti-recombinant ovPAG1 antiserum [25]. Visualization of the antigen-antibody complexes on both dot blots and Western blots was achieved with the use of alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies in an immunoblotting kit purchased from Promega (Madison, WI).

ELISA

The assay was a variation of an antibody capture assay to detect and provide a semiquantitative assay for the relative amount of ovPAGs in samples [25]. Briefly, samples (3 μ l) were applied to the bottom of a polyvinylchloride microtiter plate. The plates were washed twice with PBS and blocked with 3% BSA, and an anti-PAG antiserum (1:

50 000, each antiserum) was added to the wells. Unbound protein was removed by four washes with PBS. An alkaline solution (0.1 ml) containing alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (0.2 μ g/ml) was then added to the microplates. After washing, the complexes of antigen-primary antibody-secondary antibody were detected by adding *p*-nitrophenyl phosphate (1 mg/ml; 20 μ l) to quantitate bound enzyme [25]. The microplates were read at 405 nm to provide a measure of the amount of PAG in the original sample. The positive control in the assay was 3 μ l of the diluted 40–75% $(\text{NH}_4)_2\text{SO}_4$ fraction containing 240 ng of protein.

Hemoglobin Proteinase Assay

The hemoglobin proteinase assay was performed as described previously [24, 26]. Briefly, a 100- μ l solution containing purified ovPAG (1 μ g) and [^{14}C]methyl-hemoglobin (6 μ g; 0.04 μ Ci in 150 mM acetate buffer, pH 4.5) was incubated at 37°C for up to 48 h. Protein was then precipitated with 0.2 ml of 10% (w/v) trichloroacetic acid. Supernatant solution (0.2-ml samples) containing ^{14}C -labeled peptides liberated by the enzyme was counted in a liquid scintillation counter. Controls included negative and positive assays run in the absence or presence of porcine pepsin (Sigma), respectively.

Cloning of ovPAG Transcripts from Day 100 Ovine Placenta

PAG transcripts from Day 100 ovine placenta were cloned by reverse transcription (RT) and PCR procedures [27]. In brief, cellular RNA extracted from a Day 100 placenta was first reverse transcribed into cDNA and amplified by PCR with a pair of well-conserved primers (ovPAG forward 5'AGGAAAGAAGCATGAAGTGGCT3', ovPAG reverse 3'ATTACGAACCTGAACAAGTCCT5'; see Fig. 8). The RT-PCR products were cloned into TA cloning vectors (Invitrogen). Plasmid DNA was prepared from individual colonies and sequenced.

In Situ Hybridization

Riboprobes (cRNA) were prepared by using the Riboprobe Preparation System (Promega). Briefly, a fragment (407 base pairs [bp]) of ovPAG3 and 7 cDNA, corresponding to exons 3, 4, and 5, was amplified by PCR with two well-conserved primers (forward 5'TGGGTAACATCACCATTGGAA3', reverse 3'AAAGACTCGGACAAAAACGG5'; see Fig. 8). PCR products were subcloned into the TA cloning vectors (Invitrogen). The orientation and sequence of the inserts were confirmed by sequencing. The subcloned cDNA fragments were then transcribed in vitro into cRNA in the presence of [^{35}S]CTP. The probes (each $\sim 1.25 \times 10^9$ cpm/ μ g) were used within 3 days of preparation.

Day 100 ovine placental tissue was sectioned (14 μ m) at -18°C on an IEC cryostat (International Equipment Co., Needham Heights, MA) and mounted onto prechilled microscope slides. The sections were then fixed in 4% formaldehyde in PBS for 5 min, washed in double-strength SSC (single-strength SSC: 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 2 min, acetylated in 0.25 (v:v) acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, rinsed twice in double-strength SSC, and dehydrated sequentially through 70%, 80%, 95%, and 100% ethanol. To increase the accessibility of the target mRNA, the tissue was soaked to remove lipid membranes in 100% chloroform for 5 min,

rehydrated in 100% ethanol for 2 min and in 95% ethanol for 2 min, and air dried. In situ hybridization was performed according to procedures described previously [28] after application of 0.2 ml probe solution (4×10^6 cpm) to cover each section. Exposure was for 1–4 wk at 4°C. After development, the slides were counterstained with hematoxylin and eosin and examined microscopically.

RESULTS

Characteristics of Antisera Used in Immunodetection of PAGs during Purification

The anti-boPAG1 antiserum [1] was raised against purified boPAG1 extracted from fetal cotyledons, but it clearly recognizes an array of different ovPAG molecules [9, 29] (see Figs. 3 and 6). Presumably, different PAGs, even those belonging to different species, possess similar epitopes as a result of their sequence similarities.

The antigen used to generate the antiserum against native ovPAG1 [23] was prepared against protein fractionated as described by Zoli et al. [1] from bovine fetal cotyledons pooled from several different stages of pregnancy [23]. At the time of antiserum preparation it was believed that only a single PAG type existed. In retrospect, the antigen probably contained a mixture of potential antigens. In the context of the present experiments, the antiserum was anticipated to have relatively broad immunological cross-reactivity.

The third antiserum used was generated by immunizing rabbits against urea-solubilized, recombinant ovPAG1. Three lines of evidence suggested that this nonglycosylated product was largely denatured. First, when the urea was removed from the preparation, much of the protein precipitated from solution; second, the protein that remained soluble eluted with the void volume on a Superose 12 HPLC column; third, the protein failed to bind to a pepstatin affinity column (data not shown). The antiserum raised against this recombinant protein was likely, therefore, to recognize certain epitopes possibly present on improperly folded but not native protein and to have some value in recognizing a range of PAGs on Western blots.

When used in combination, it seemed likely that these antisera would be capable of detecting a broad array of PAGs produced by Day 100 placental tissue.

Fractionation of ovPAGs from Cultured Placental Explants

Proteins that had been released by explants of Day 100 placental tissue during an 8-h culture in serum-free medium were initially fractionated by addition of ammonium sulfate (Fig. 1). Little material that cross-reacted on Western blots with any of the anti-PAG antisera was detected in either the 20% or 40% ammonium sulfate fractions, which contained 0.29 g and 5.80 g of protein, respectively. The protein (11.5 g) that was precipitated at between 40% and 75% ammonium sulfate saturation contained all but a trace of the immunopositive material (data not shown).

After dialysis against 0.1 M sodium acetate (pH 5.5), a portion of this protein fraction (405 mg) was passed twice through a Blue Sepharose column to remove much of the serum proteins, particularly serum albumin, that contaminated the preparation. Bound protein, which could be subsequently eluted in 0.5 M NaCl, contained no ovPAG1-immunoreactive material as determined by immunoblotting. Instead, the PAGs were all present in the initial breakthrough fraction (243 mg).

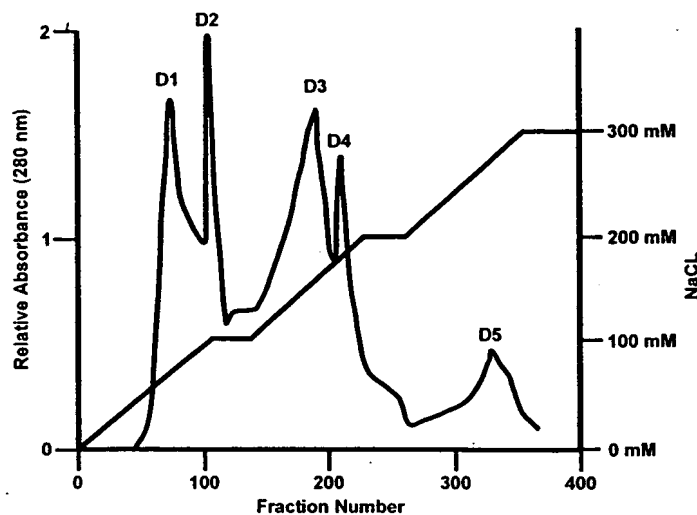


FIG. 2. Anion-exchange chromatography of protein fractionated by precipitation with ammonium sulfate on a column of DEAE-Sepharose. Proteins precipitated with 40–70% saturated ammonium sulfate were resuspended in 10 mM Tris (pH 8.0), dialyzed, and then passed three times through a Blue Sepharose column. The proteins in the final breakthrough from the Blue Sepharose column were concentrated, loaded onto a DEAE-Sepharose column (25 × 92 cm), and washed extensively with 10 mM Tris, pH 8.0. The column was eluted by a combination of step gradient and linear gradient of 0–300 mM NaCl in 10 mM Tris, pH 8.0. The eluate was collected in 9.5-ml fractions whose content of protein was assessed by their absorbance at 280 nm. Each fraction was analyzed for immunoreactivity by using dot blotting and ELISA. Five pools were generated, and pool 1 (D1) was divided into two subpools (D1A and D1B).

Anion-exchange chromatography on DEAE-cellulose at pH 8.0 was then used to purify the PAGs further (Fig. 2). The unbound, flow-through fraction contained about one quarter of the 243 mg protein loaded, but no detectable material that reacted with the antisera (Fig. 3, A and B, lane 3). Subsequent elution with a gradient of NaCl yielded five major pools (Fig. 2; D1–D5). Pool D1 was arbitrarily divided into two subpools (D1A, leading peak; D1B, the trailing shoulder). Both contained material that reacted strongly with the antisera to boPAG1 (Fig. 3C, lanes 4 and 5) and anti-recombinant ovPAG1 (Fig. 3D) but relatively weakly with native ovPAG1 antiserum (Fig. 3B).

In contrast, pool D3 reacted most strongly with anti-native ovPAG1 antiserum and not at all with anti-native boPAG1 (Fig. 3B, lane 7). Although pools 2, 4, and 5 all contained immunoreactive protein (Fig. 3, B–D, lanes 6, 8, and 9, respectively), these were not studied further. Instead, the effort was to purify the PAGs in pools D1 and D3.

Purification of PAGs from Pool D1A

Pool D1A (fractions 50–71, Fig. 2; 19.4 mg protein) was subjected to high-performance cation-exchange chromatography on a Mono S column. All immunoreactive material bound to the column, and there was very little protein (< 1 mg) in the flow-through. Salt elution provided three further pools of PAGs (S1, 4.4 mg; S2, 5.7 mg; S3, 5.0 mg; Fig. 4A). After electrophoresis, a broad band of Coomassie blue-staining material was evident in each pool (Fig. 4B) that cross-reacted with the antiserum to boPAG1 (Fig. 4C).

Further fractionation of a portion (2.8 mg) of pool S1 on a Mono P column, in which proteins are separated according to their isoelectric points, gave five further pools of protein (S1P1–S1P5; pI 5.54, 5.19, 4.06, 4.00, and 3.56, respectively) (Fig. 5). Each contained a protein of apparent

FIG. 3. Electrophoretic analyses and Western immunoblotting of proteins fractionated by DEAE-Sephacrose. Samples were subjected to electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. Lane 1, molecular weight standards (scale on left $\times 10^{-3}$); 2, total proteins initially loaded on the DEAE-Sephacrose column; 3, proteins in the unbound fraction. Lanes 4 to 9 correspond to pooled materials of D1A, D1B, and D2 through D5, respectively (see Fig. 2). In A, proteins (10 μ g loaded) were visualized by staining the gel with Coomassie brilliant blue. B, C, and D are Western blots (2 μ g loaded per lane) analyzed with either anti-native ovPAG1 (B), anti-native boPAG1 (C), or anti-recombinant ovPAG 1 antisera (D).

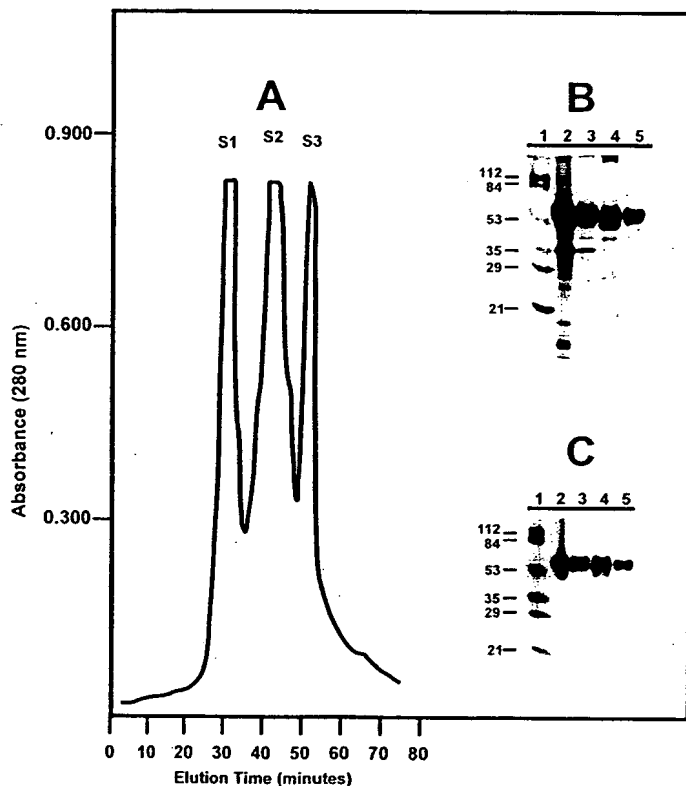
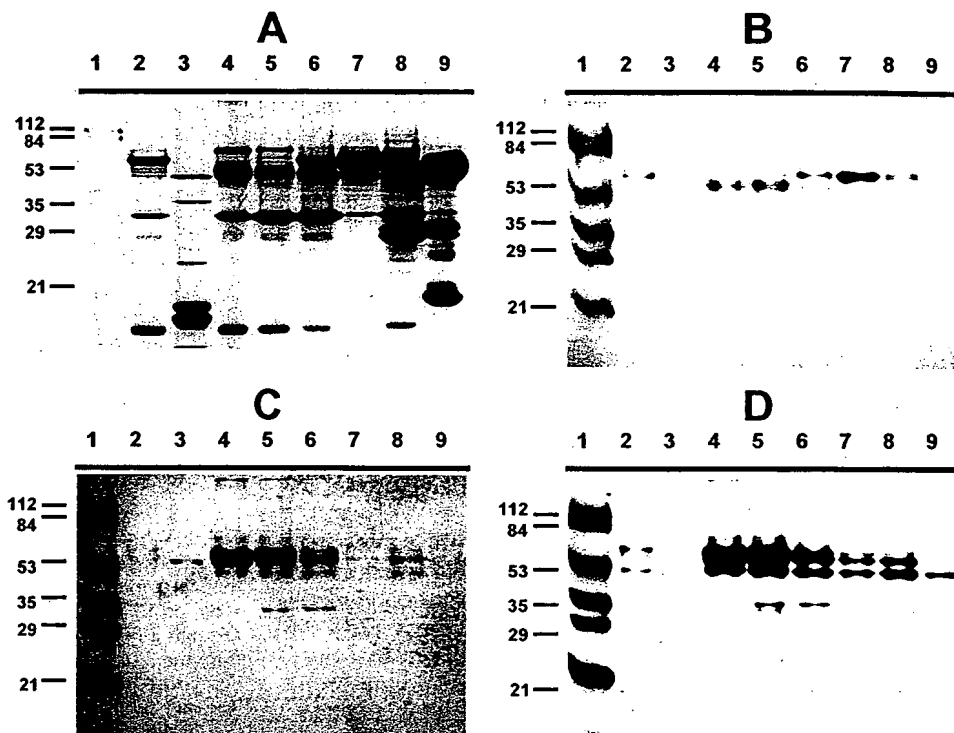


FIG. 4. Analysis of proteins from DEAE-Sephacrose fraction D1 by Mono S chromatography. A) Proteins from the D1 fraction were loaded onto a Mono S column. The column was washed extensively with 20 mM sodium acetate (pH 5.0) and eluted with a gradient of 0.1 M NaCl in 20 mM sodium acetate (pH 5.0) at a flow rate of 1 ml/min. The eluate (1 ml/fraction) was assayed for ovPAG by dot blotting and ELISA. The fractions in pools S1, S2, and S3 were combined separately and analyzed by SDS-PAGE (B and C). Lane 1, molecular weight standards (scale on left $\times 10^{-3}$); 2, total protein initially loaded on the Mono S column; lanes 3, 4, and 5 correspond to pooled materials of S1, S2, and S3. Proteins were visualized either by staining with Coomassie blue (10 μ g/sample) (B) or by immunoblotting (2 μ g/sample) with anti-native boPAG1 (C).

mass ~ 65 kDa (Fig. 6, lanes 7–11). This band cross-reacted weakly with anti-ovPAG1 (Fig. 6B, lanes 7–11) but strongly with anti-recombinant ovPAG1 (Fig. 6D). Fractions S1P1 and S1P2 were also positive against anti-boPAG1 (Fig. 6C).

Material in two of these pools (S1P2 and S1P4) was selected for sequencing. The two (designated ovPAG₆₅ in Fig. 7) were identical over the first 21 amino acids (aa) of amino-terminal sequence.

Pools S2 and S3 from Figure 3 were also fractionated further on the Mono P column. Only two peaks were generated for each sample (data not shown). The proteins in the two peaks (S2P1, 0.8 mg; S2P2, 0.02 mg) from the S2 pool had isoelectric points of 5.68 and 4.0, respectively, and an apparent molecular mass of 61 kDa (Fig. 6A, lanes 12 and 13). A band of slightly higher mobility (apparent mass 55 kDa), which cross-reacted strongly with the anti-

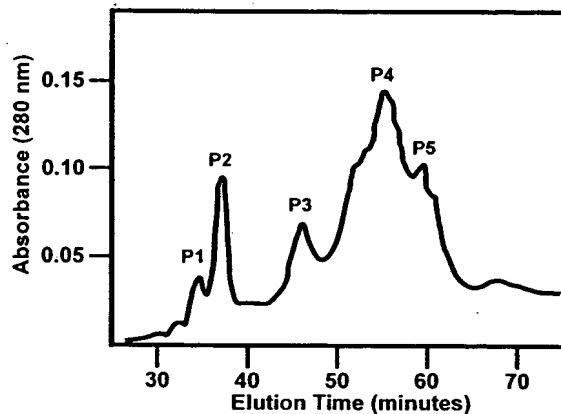


FIG. 5. Isoelectric point analysis of ovine placental proteins in fraction S1 by chromatography on a Mono P column. A) Proteins from the Mono S fraction S1 (Fig. 4) were loaded onto a Mono P column equilibrated with start buffer (0.025 M bis-Tris, pH 6.3). The column was eluted with polybuffer 74 (Pharmacia) at a flow rate of 1 ml/min. Samples were collected as five pools (P1, P2, P3, P4, and P5, respectively).

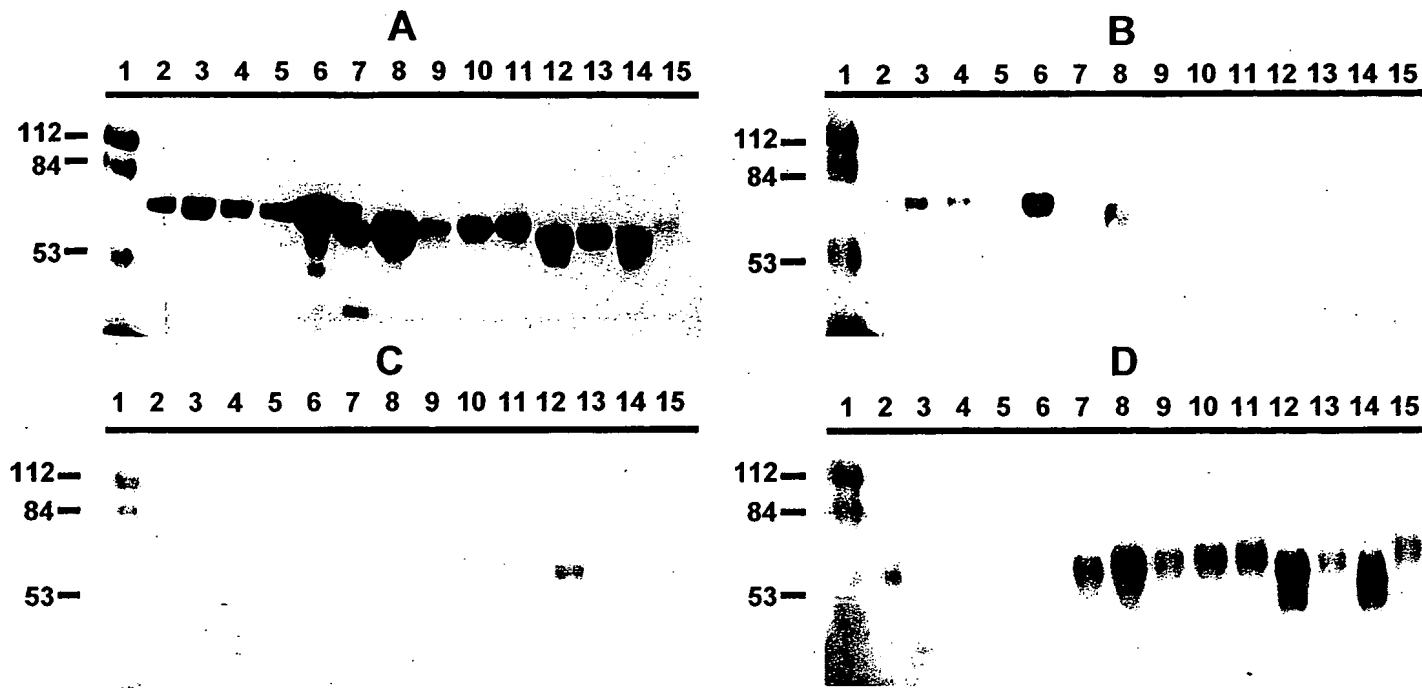


FIG. 6. Electrophoretic and Western immunoblot analyses of proteins fractionated by Mono P chromatography. Samples were subjected to electrophoresis on polyacrylamide gels containing 0.1% SDS. Lane 1, molecular weight standards (scale on left $\times 10^{-3}$). Lanes 2–6 were samples derived by further fractionation of the DEAE-Sepharose D3 pool (see Fig. 2). This was first separated into two fractions by Mono S chromatography (data not shown). Only the Mono S fraction 1 contained immunoreactive materials and was further separated by use of Mono P chromatography into three peaks. Lanes 2 and 3 correspond to the leading and trailing ends of Mono P peak 1; lanes 4 and 5 to the leading and trailing edges of peak 2; lane 6 represents peak 3 from the Mono P column. Lanes 7–11 correspond to fractions P1 through P5 from Figure 5, respectively. Fraction S2 (Fig. 4) was separated into two subfractions by Mono P chromatography; lanes 12 and 13 represent subfractions. Fraction S3 (Fig. 4) was also separated into two fractions by Mono P chromatography; lanes 14 and 15 were samples from subfractions 1 and 2, respectively. In A, gels were stained by Coomassie blue and were loaded with 5 μ g protein per lane. B–D were immunoblots containing 0.5 μ g protein per lane. These immunoblots were developed with either anti-native ovPAG1 (B), anti-native boPAG1 (C), or anti-recombinant ovPAG1 (D).

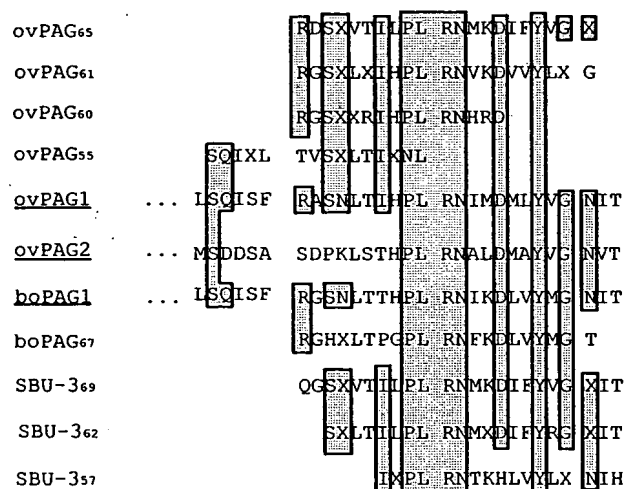


FIG. 7. Comparison of the NH₂-terminal amino acid sequences of four purified ovPAGs with other PAG-related proteins that were purified from placental tissues and with three PAGs that were cloned but not purified. OvPAG₆₅ and ovPAG₆₁ correspond to the samples shown in Figure 6, lanes 8 and 12, respectively. OvPAG₅₅ corresponds to the lower band and ovPAG₆₀ to the slower-mobility band in Figure 6, lane 14. The subscript for each ovPAG denotes its apparent molecular mass (in kDa). The purified PAGs were subjected to microsequencing. Sequences obtained were compared with those of three glycoproteins isolated by immunopurification on the SBU-3 monoclonal antibody [20], the boPAG₆₇ sequence reported by Zoli et al. [1], and the sequences inferred for ovPAG1 [9] and ovPAG2 [18] from their cDNA. Regions of strongest sequence conservation are shaded. X indicates a blank cycle and probably corresponds to a glycosylated asparagine residue. The cDNA for ovPAG1 (GenBank accession no. M73961), ovPAG2 (U30251), and boPAG1 (M73962) encode both the signal sequence and the propeptide. The existence of additional amino acids at the amino termini is represented by dots.

FIG. 8. Nucleotide sequence of cDNA clones ovPAG3 and ovPAG7 identified by RT-PCR from RNA isolated from Day 100 cotyledonary RNA. The arrows at the beginning and end of the sequences correspond to those chosen to provide probes for the initial PCR amplification of reverse-transcribed RNA. The two internal arrowed sequences are those selected to provide the probe used for in situ hybridization. The two solid rectangles mark the initiation and stop codons, respectively.

Consensus	AGGAAAGAGCATGAAGTGGCTTGTGCTCTYGGGCTGGTGGCTTCTCAGAGTGCCATKTYAAAGTACCTCTAAGGAGATTGAAGACCA	90
ovPAG7C.C.....T.T.....	90
ovPAG3T.T.....G.C.....	90
Consensus	TGAGAAAAACCTCAGTGGAAATACATCTGTAACAATGTCTTGAAGGATATCTTATAGACTGCCCCAGATTCTTTTCGTGATTCAA	180
ovPAG7G.....	180
ovPAG3A.....	180
Consensus	ATGTAACATTCTCCCATTTGAGAAACATGAAAGATATATTCTACGTGGGTAAACATCACCATTGGAACACCTCCTCMGGAATTCAGGTTG	270
ovPAG7C.....	270
ovPAG3A.....	270
Consensus	TCTTTGACASRCTTCATCTGACTTGTGGGTGGCTCCATCTTTTGAACAGCTCAACCTGTTTACACKTGTAGGTTTCARACATCGCC	360
ovPAG7GCA.....G.....T.....A.....	360
ovPAG3CAG.....C.....G.....	360
Consensus	AGTCTTCCACCTTCCGGHYRCSAATAAGACATCTCKGGATCAYSTATGGAGCTGGGAAATGAAGGAGTTGTTGTTTCATGACACAGTTC	450
ovPAG7ACAA.G.....T.....CC.....C.....C.....	450
ovPAG3CTTG.C.....G.....TG.....A.....	450
Consensus	GGATTGGGACCTGTGAAGTATGACAGCCATTGCTTAAGCTGGYRGAATHYGGTTTTRWGSACAGAMRATTTGATGGYRCTTGG	540
ovPAG7T.....T.....A.....CA.....AC.....CAT.G.....AG.....CA.....	540
ovPAG3C.....C.....G.....TG.....CT.....TGA.C.....CA.....TG.....	540
Consensus	GCTTGAACATCCCAACMCACTCTCTGAAAACCAWSSATCTTTGACAAGCTGAARARTSAAGGTGCCATTTCTGAGCCTGTTTTTG	630
ovPAG7GA.A.....G.....AGTGG.....A.G.C.....	630
ovPAG3AC.T.....T.....TCCCC.....G.A.G.....	630
Consensus	CCTTCTACTTGAGCAAGAYGAGCAGGARGGCAGTGTGTGATGTTTGGTGGKGTGGACACASTTACTACAAGGARAGCTCAACTSSS	720
ovPAG7C.....G.....G.....G.....G.....C.....G.....CCC	720
ovPAG3T.....A.....A.....T.....T.....A.....A.....GGG	720
Consensus	TACCATTGRTCAAGCRGYGACTGGAGTGTACRTGTGACMGCATYWCATGARAAGAGAGGTATTGCTTGTCTGAYGGCTGYRRKG	810
ovPAG7G.....G.AT.....A.....A.....A.....C.....TT.....G.....T.....TGAT.....	810
ovPAG3A.....A.GC.....G.....G.....G.....A.....CA.....A.....C.....CAGG.....	810
Consensus	CCCTKRTGGACACYGGKCATCAWYATCCAMGGCCAGGAAGACTGATCGATACRATACARAAGCTSATWGGCWCRCWCHACRRGWT	900
ovPAG7TT.....T.GG.....TT.....G.....A.....C.....T.....T.....GA.....A.....GA.AT.....	900
ovPAG3GG.....C.....TT.....CA.....A.....A.....G.....G.....G.....A.....A.....AT.....C.....AG.GA.....	900
Consensus	YCAMGCACTAYRTTYCATGTTCTGCGGTCAATACTCTGCCCTTATTATCTTACCATCAATGGCATCAACTACCCAGTGCAGCTCAAG	990
ovPAG7	T.A.....CA.T.....	990
ovPAG3	C.T.....TG.C.....	990
Consensus	CCTACATCTCAAGGGTTTACAGGCCACTGCTATACGCGCTTTAGAGCGAAAGAGTGAGGACATCTACAGAGTCTGGGTCTGGGTG	1080
ovPAG7C.....	1080
ovPAG3T.....	1080
Consensus	AYGCTTTCAGGCTGTATTCTCAGTCTTTGATCGAGGAMATGACAGGATTGGHCTSGCASCSSGRCARRTAAATGCTTGGACTTGTTC	1170
ovPAG7C.....C.....A.....A.....G.....C.....ATG.....	1170
ovPAG3T.....T.....C.....C.....G.....GGA.....	1170
Consensus	AGGAA	1175
ovPAG7	1175
ovPAG3	1175

recombinant ovPAG1 antiserum, was also detected in the S2P1 material (Fig. 6D). However, sequencing of the total material in S2P1 was consistent with the presence of only a single polypeptide. If a mixture of PAGs had been present, more than one signal might have been evident during at least some of the cycles. The sequence (ovPAG₆₁) obtained was again unique.

The two Mono P peaks from the S3 pool (S3P1, pI 5.54, 0.24 mg; S3P2, pI 4.00, 0.2 mg) contained proteins that differed in apparent mass (Fig. 6, lanes 14 and 15). S3P1 itself also contained at least two bands of material (60 kDa and 55 kDa) when it was analyzed electrophoretically (Fig. 6, lane 14). Each was subjected to "off-blot" analysis. The two (ovPAG₆₀ and ovPAG₅₅) differed markedly in their amino-terminal sequences (Fig. 7). The polypeptides in fraction S3P2 have not been analyzed further.

Further Analysis of Polypeptides in Pool D3

Again the sequential use of chromatography on Mono S and Mono P columns provided fractions that appeared relatively homogeneous in composition (Fig. 6, lanes 2–6). These protein bands (mass ~70 kDa) reacted weakly with anti-native ovPAG1 but not with the other two antisera. Sequencing failed to reveal the presence of PAG. Instead, the data indicated that most of the protein in pool D3 was serum albumin (data not shown) whose signal was strong enough to mask that of any PAGs.

Purified PAGs Lack Proteolytic Activity Toward Denatured Hemoglobin

In order to examine whether any of the PAGs so far purified possessed proteolytic activity, 1 µg of each was incubated with 6 µg [¹⁴C]methyl-hemoglobin for as long as 48 h at 37°C. No hydrolytic activity was evident in any of the preparations. By contrast, 1 µg of pepsin, the positive control, converted the majority of the protein to trichloroacetic acid-soluble peptides within 1 h under identical conditions.

Cloning of PAG Transcripts from Day 100 Placenta

In order to isolate transcripts that possibly matched some of the ovPAGs that had been purified (Fig. 7), mRNA from Day 100 placental tissue was reverse transcribed and subsequently amplified by PCR. The oligonucleotides used for PCR represented sequences just 5' to the ATG start codon and 3' to the stop codon in regions well conserved in all PAG transcripts so far cloned [9–12, 18, 19]. A total of 25 cDNA, distinct from but closely related to ovPAG1 and ovPAG2 in sequence, were sequenced, and these could be classified into seven different groups. They were named ovPAG3 to ovPAG9 based on the order in which they were sequenced to completion (Genbank accession numbers 494789 to 494795, respectively). Two of the seven cDNA (ovPAG3 and ovPAG7) encoded a polypeptide that contained sequence corresponding to the amino terminus of

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FIG. 9. The inferred amino acid sequences of ovPAG3 and ovPAG7. The inferred signal sequence of 15 aa is shown. The shaded rectangle corresponds to the region of apparent identity between ovPAG3, ovPAG7, and ovPAG₆₅ (from Fig. 7). The asterisks mark asparagines that are potentially glycosylated. The open rectangles designate the sequences immediately surrounding the two aspartic acid residues (D) that are normally involved in catalysis. The signal sequence of 15 aa is numbered separately. The propeptide region is between the end of the signal sequence and the shaded rectangle.

ovPAG₆₅ shown in Figure 7. Despite this similarity at their 5' termini (Fig. 8), the two were clearly distinct in nucleotide sequence (Fig. 8), and it was clear that they encoded different proteins (Fig. 9). None of the other cDNA (ovPAG4, 5, 6, 8, and 9) encoded any of the protein sequences shown in Figure 7.

The lengths of the polypeptides encoded by ovPAG3 and ovPAG7 (each 380 aa) were the same, but the sequences

showed only 84% identity overall (Fig. 9). After the signal peptide (15 aa) and propeptide (38 aa) regions had been removed, the mature ovPAG3 and ovPAG7 proteins had theoretical molecular weights of 36 281 and 36 680, respectively. The additional mass of the proteins is presumably the result of posttranslational processing, particularly of the addition of carbohydrate, as has previously been reported for ovPAG1 [9, 29]. Both ovPAG3 and ovPAG7 possess four conserved asparagine residues in the context Asn X Thr/Ser, which could be targets for *N*-glycosylation. In addition, ovPAG3 contains a fifth such site (Fig. 9). Edman sequencing of ovPAG₆₅ (Fig. 7) provided no signal on cycles 4 and 21, which correspond to Asn57 and Asn74 on both ovPAG3 and ovPAG7, suggesting that the residues at these positions are indeed glycosylated.

Aspartic proteinases that are enzymatically active characteristically possess conserved sequences of amino acids around the two catalytic aspartic acid residues that face each other across the peptide-binding cleft separating the two lobes of the molecule (unshaded boxes in Fig. 9). In ovPAG3, glycine in the usually conserved triad DTG in the amino-terminal lobe [9, 19, 21] is replaced by an alanine. A similar mutation is believed to be inactivating in the case of boPAG1 [9, 12]. In ovPAG7, the same DTG triad is replaced by DST (Fig. 9) and again is not anticipated to support enzymatic activity.

Localization of ovPAG3 and ovPAG7 mRNA to Trophoblast Binucleate Cells

In situ hybridization was performed on sections of Day 100 placentomes with probes representing relatively poorly conserved regions of ovPAG3 and ovPAG7 transcripts (Fig. 8). Positive hybridization signals were restricted to the scattered binucleate trophoblast cells (Fig. 10) that make up about 25% of the cells of trophoderm [30]. This localization is clearly observed near the tips of the chorionic villi close to the edges of the placental villi (Fig. 10A) but is also evident in the meshwork of chorionic villi in the central region of the placental villi (Fig. 10B). There, the villi

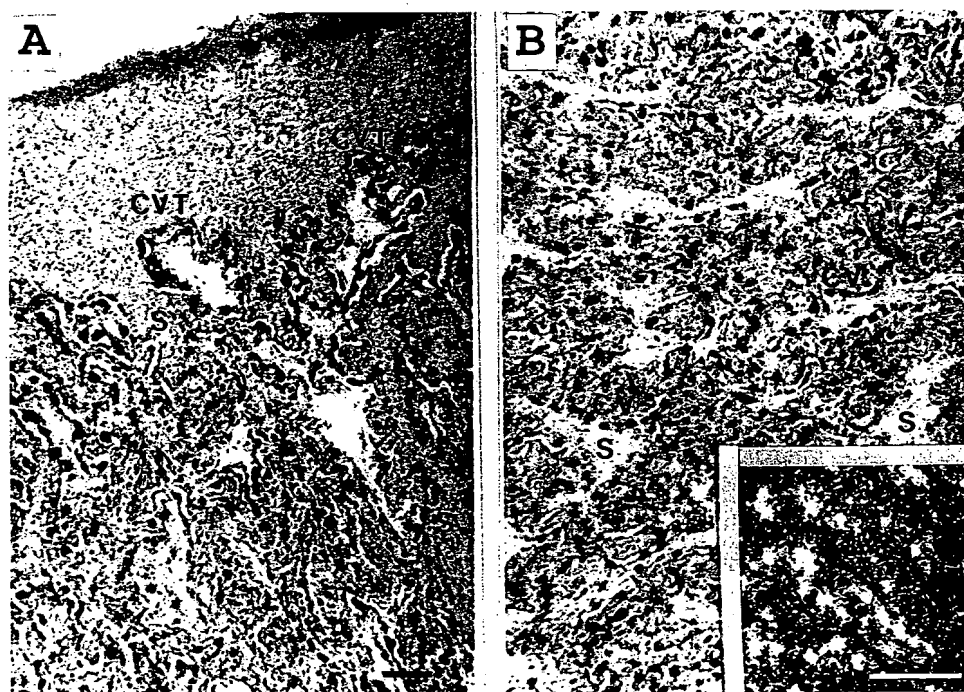


FIG. 10. Localization of the mRNA for ovPAG7 and ovPAG3 in Day 100 ovine placental tissue by in situ hybridization analyses. In situ hybridization was performed with ³⁵S-labeled probes on transverse sections across a placental villi removed from a sheep at Day 100 of pregnancy. Two sections are shown. A) A section near the edge of the placental villi showing the tips of cotyledonary villi with the strongly positive binucleate cells heavily labeled with silver grains from the ovPAG7 probe. B) A section within the central region of the placental villi showing cross sections of cotyledonary villi as islands between thin septae of maternal caruncular tissue. Again, all the label from the ovPAG7 probe is confined to binucleate cells. The inset shows a similar section hybridized to an ovPAG3 cDNA but photographed under dark-field illumination. Magnification is about twice that in the main figure. Silver grains appear white. CVT, cotyledonary villous tips; CV, cotyledonary villi in cross section; S, caruncular septae protruding between cotyledonary villi. Bars = 100 μm.

appear as islands separated by thin septa of maternal tissue [30, 31]. This restricted distribution of PAG mRNA appears identical to that observed for ovPAG1 [9] and unpublished results) and different from that shown by ovPAG2, whose expression is strongest in mononucleated cells [18].

DISCUSSION

These data confirm earlier suspicions that the PAGs are the products of many genes [10, 21]. In our initial studies, we had observed that anti-ovPAG1 antisera could recognize several molecules in medium from cultured trophoblast, these molecules ranging in apparent mass from 45 kDa to 70 kDa; but we attributed much of the complexity to heterogeneous processing of an initial single form [9]. As other PAGs were discovered, this simplistic view had to be amended. Here, a combination of protein purification, microsequencing, and cDNA cloning has provided evidence for at least 12 different ovPAGs at around Day 100 of pregnancy in the ewe. The fact that several immunoreactive pools of protein from ion-exchange chromatography were not chosen for further analysis, and that only 25 of several hundred positive cDNA clones were sequenced, suggests that many more PAGs remain to be discovered. The fact that the PAGs recognized by the SBU-3 monoclonal antibody [20] were all different from the ones reported here and from ovPAG1 [9] and ovPAG2 [18] tends to confirm this likelihood.

Even amino acid sequencing can underestimate PAG complexity. Here we described two distinct cDNA (Figs. 8 and 9) each of which could have encoded one of the PAGs (ovPAG₆₅) that had been column purified (Fig. 7). In fact, ovPAG3 and ovPAG7, although expressed simultaneously and in the same binucleate cell type, have only 84% sequence identity over their full lengths. Their similarities at their amino termini could have arisen by a recent gene-conversion event.

These interpretations of the data are not meant to imply that posttranslational modifications do not also contribute to PAG heterogeneity. These molecules are glycosylated at asparagine residues and probably at serines and threonines as well [29]. Some, at least, are phosphorylated [29], and the loss of higher molecular weight forms during pulse-chase experiments suggests that proteolytic processing also occurs [9, 29].

Our data confirm that even an antiserum raised against a homogeneous PAG product binds several different PAGs on Western blots (Figs. 3 and 6). On the other hand, not all PAGs can be recognized on such blots by a single antiserum. Such antigenic variability is not surprising, as the molecules demonstrate a considerable range of sequence identities. Antisera may show rather more selectivity in screening of cDNA libraries than in blotting experiments. The anti-PAG antisera of Zoli et al. [1, 23], for example, were remarkably specific for fusion proteins encoded by PAG1 inserts [9]. A second antiserum recognized boPAG2 fusion proteins exclusively [11]. Conceivably, much of the antigenicity of the fully processed molecules is provided by carbohydrate, absent on bacterial fusion proteins. The work of Atkinson et al. [20] predicted that trophoblast binucleate cells, which synthesize many PAGs, express a structurally unusual carbohydrate antigen that is specifically recognized by the SBU-3 monoclonal antibody.

The PAGs are clearly major secretory products of the ovine placenta, although their exact contribution cannot be easily assessed from our purification scheme because the

starting solution was undoubtedly contaminated with serum leached from the tissue capillaries of the cotyledonary explant cultures. Indeed, if the initial Blue Sepharose step was not included in the purification protocol, albumin contaminated, to some extent at least, every subsequent protein pool (data not shown). Nevertheless, except for some additional albumin in pool D3, which had escaped entrapment on the Blue Sepharose, PAGs seemed to be the dominant components of the fractions pooled from the initial DEAE-cellulose column steps (Fig. 2) and subsequent fractionation (Fig. 4).

None of the four ovPAGs examined had proteolytic activity when tested against denatured hemoglobin. One explanation is that, like renin, they have narrow specificity. However, the lack of consensus peptide sequence in the catalytic centers of ovPAG3, ovPAG7, and several other PAGs identified earlier [12, 19, 21] strongly suggests that these particular molecules do not serve a proteolytic function. Unfortunately there is little to indicate what actual roles these curious molecules might play [12, 32]. A region of propeptide, which in pepsinogens fits tightly into the substrate-binding cleft and prevents catalytic activity until it has been removed [33], has been excised from the PAGs, presumably by some active proteinase, by the time they have been purified. Although this processing is not uniform (see Fig. 7), the event seems not to be random. In three of the ovPAG group purified here, for example, proteolytic cleavage of the propeptide appears to be associated with a relatively conserved ISF↓RG/DS sequence, where the arrow indicates the site of peptide bond cleavage (Fig. 7). Members of the SBU-3 group, on the other hand, are processed at a different site.

Removal of the propeptide exposes the cleft formed between the two lobes of the molecules [12] and might well provide the PAGs the ability to associate with peptides without hydrolyzing them [32]. It seems unlikely that the PAGs are mere biological curiosities, as they have been found in all hoofed animals so far examined and encompass an evolutionary distance that is probably greater than 80 million years [12].

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Pregnancy-associated alpha₂-glycoprotein (α_2 -PAG): development of a sensitive enzyme-linked immunoassay and comparison of serum concentrations in adults and children

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Key words: Enzyme immunoassay; α_2 -PAG; Estrogen; Androgens

Summary

Using a newly-developed and very sensitive enzyme-linked immunoassay technique, we have measured circulating α_2 -PAG concentrations in 270 adults and 181 children. In adults, levels increased with age in both sexes and were significantly higher in women in each age category studied. In contrast, there were no significant differences between the sexes in children and no changes with age between 1 and 14 years. At puberty, α_2 -PAG concentrations increased in girls and decreased in boys. The increase in girls is consistent with the known stimulatory effects of oestrogen on α_2 -PAG production. We discuss the possibility that androgens may have an analogous inhibitory effect in males.

Introduction

Pregnancy-associated alpha₂-glycoprotein (α_2 -PAG) is a high molecular weight glycoprotein (mol wt 360 000) which is present, in small amounts, in the serum of all normal individuals [1]. Synthesis of α_2 -PAG is stimulated by oestrogen [2,3] and levels are markedly elevated in women taking oral contraceptives [4], in men undergoing stilboestrol therapy for the treatment of prostatic carcinoma [2] and during pregnancy [5]. The biological role of α_2 -PAG is unknown although it has

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been shown to have immunosuppressive effects *in vitro* [6,7] and perhaps *in vivo* [8]. Increases in serum α_2 -PAG concentrations have been described in a variety of disease states including malignancies [2,9,10], rheumatoid arthritis [11] and psoriasis [12]. However, conflicting results have been obtained in such studies which generally fail to take into account the age-dependent increase in α_2 -PAG concentrations and the significantly different mean levels in males and females in their selection of suitable control groups [1].

In order to provide a basis for the study of disease conditions, we have used a new and very sensitive immunoassay to establish the normal range of α_2 -PAG concentrations in plasma from adult donors. We have also measured circulating α_2 -PAG concentrations in children and demonstrate striking changes in these levels in both sexes at puberty.

Materials and methods

Enzyme immunoassay

Solutions:

Coating buffer	15 mmol/l sodium carbonate, 35 mmol/l sodium bicarbonate, pH 9.6; 200 mg/l thiomersal
Incubation medium	PBS (0.15 mol/l NaCl, 10 mmol/l sodium phosphate buffer, pH 7.2), 10 μ g/l bovine serum albumin (BSA), 500 μ l/l Tween-20
Washing solution	PBS, 1 ml/l Tween-20
Chromogen buffer	0.15 mol/l citrate/phosphate buffer, pH 5.0
Stopping solution	4 mol/l H_2SO_4

Preparation of rabbit anti-human α_2 -PAG

New Zealand white rabbits were each injected intramuscularly (IM) with 50 μ g of purified α_2 -PAG (supplied by Dr. Hans Bohn, Behringwerke AG) emulsified in a final volume of 1 ml (1:1, PBS: Freund's complete adjuvant). After 4 wk, the animals were boosted with a further 1 ml of purified α_2 -PAG (270 μ g) delivered intravenously (0.4 ml) and IM (0.6 ml). One week later, the specificity and titre of antibody in a test bleed were established by immunoelectrophoresis and immunodiffusion.

The serum obtained from these rabbits was then absorbed with normal male serum containing < 100 μ g α_2 -PAG/l, and an immunoglobulin fraction prepared by chromatography on Sephacryl S300. Fractions containing anti- α_2 -PAG activity were pooled, concentrated to the original sample volume of the column (6 ml) and stored in batches of 200 μ l at $-20^\circ C$.

This serum gave reactions of complete identity in double diffusion experiments with commercially-available rabbit anti-human α_2 -PAG antisera (Dako, Behringwerke AG) and was monospecific as assessed by immunodiffusion against electro-

phoretically-separated proteins from late pregnancy serum. It showed no cross-reactivity with the normal male serum used for absorption.

Assay procedure (see Fig. 1)

Coating Microelisa plates (Dynatech, M129A) were coated overnight at room temperature with 200 μ l/well sheep anti-human α_2 -PAG (Seward), diluted 1:1000 in coating buffer (= 25 mg protein/l). The wells were then aspirated and washed three times, prior to the addition of sample material.

Antibody-antigen binding stages

Each incubation described below was carried out at 25°C for 90 min and was terminated by washing the plates three times. All dilutions were carried out using incubation medium. 200 μ l/well of the following solutions were used:

- 1st incubation suitable dilutions of sample material
- 2nd incubation rabbit anti-human α_2 -PAG (IgG fraction) at a dilution of 1:2500 (= 10 mg protein/l)
- 3rd incubation goat anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma) at a dilution of 1:7000.

Peroxidase reaction

Hydrogen peroxide (0.3 g/l) and *o*-phenylenediamine (10 mmol/l) were mixed

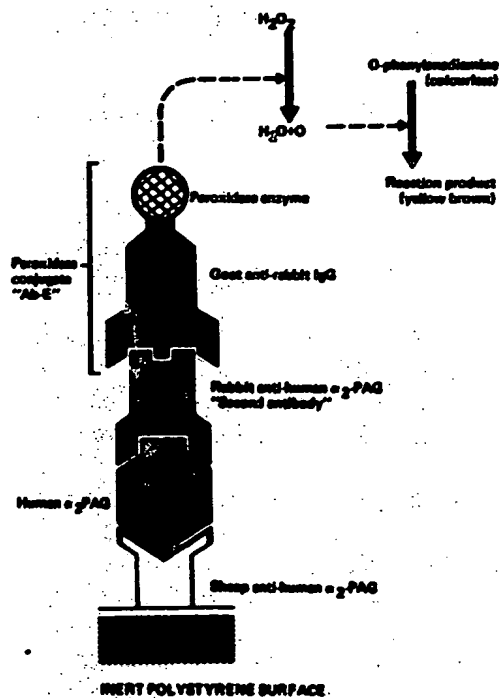


Fig. 1. Diagrammatic representation of the ELISA technique described in 'Materials and Methods'.

with chromogen buffer immediately before use and 200 μ l of this substrate solution added to each well. The peroxidase reaction was left to proceed for 30 min in the dark at room temperature and stopped by the addition of 50 μ l of 4 mol/l H_2SO_4 .

Determination of results

The final absorbance was measured spectrophotometrically at 486 nm using a Kontron SLT 210 Automatic Analyser. A standard curve of samples containing 3–1000 μ g α_2 -PAG/l was prepared by dilution of standard sera provided by Behringwerke AG and included in each assay. The concentration of α_2 -PAG in the samples was determined directly from this curve.

Blood samples

Adults Plasma was prepared from venous blood samples collected into potassium/EDTA to prevent coagulation. Donors, all of whom were apparently healthy, included medical and laboratory staff, medical and nursing students and individuals attending clinics at Aberdeen Royal Infirmary (ARI) for routine medical examinations. A total of 270 suitable samples were selected and collated by the Department of Haematology, ARI; women who were either pregnant or taking oral contraceptives were excluded from the study.

Children A total of 181 routine serum samples from the Royal Aberdeen Children's Hospital were collected by the Department of Chemical Pathology, ARI.

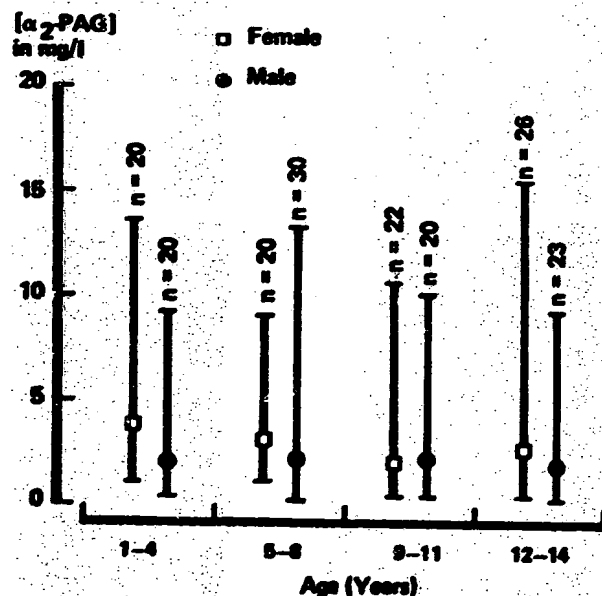


Fig. 2. α_2 -PAG concentrations in children aged between 1 and 14 yr.

In this case no attempt was made to collect a 'normal range' and only the age and sex of the children were determined. A smaller 'control' group of 40 serum samples from epileptic children who were thought to be otherwise healthy was also collected. These blood samples had been sent to the Department of Chemical Pathology for measurement of anti-epileptic drug levels.

All serum and plasma samples were stored at -20°C prior to assay.

Statistical analysis

The distribution of α_2 -PAG concentrations in both adults and children showed a marked positive skew. In order to give comparable levels of variance between groups, logarithmic transformation of the results was therefore carried out prior to statistical analysis using the unpaired Student's *t* test.

The data was also analysed, without transformation, using a Mann-Whitney rank

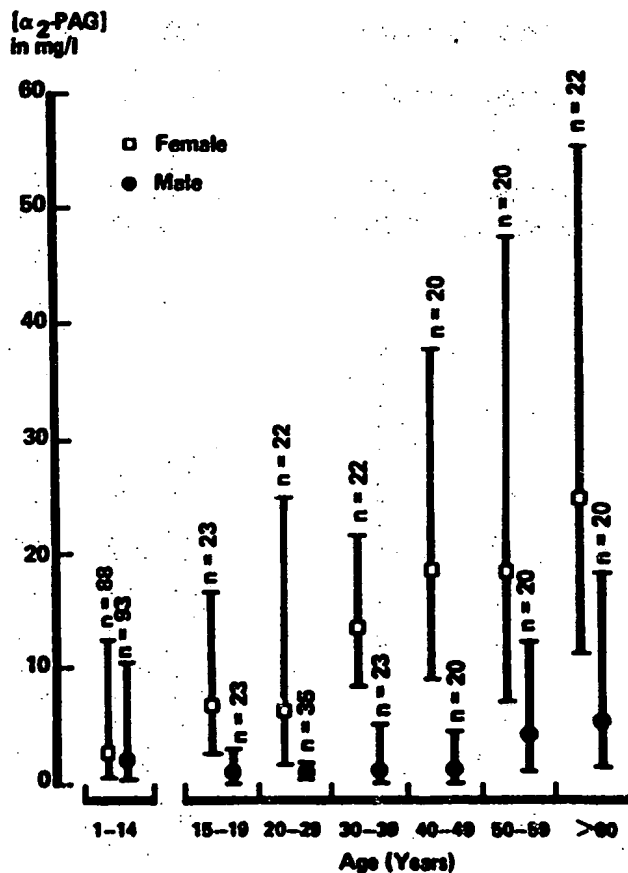


Fig. 3. Effect of age on α_2 -PAG concentrations in adults: concentrations in children are shown for comparison.

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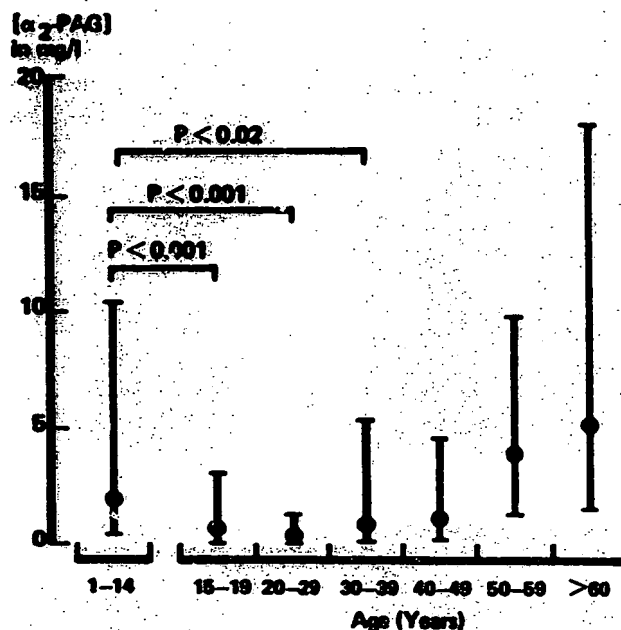


Fig. 4. Changes in α_2 -PAG concentrations with age in males showing the statistically significant difference between children and young adults.

sum test and the same comparisons were found to be statistically significant ($p < 0.05$) by both methods.

For ease of presentation, each mean and SD shown in Figs. 2-4 was derived from logarithmically transformed data.

Results

Assay precision and sensitivity

Samples for precision studies were prepared by making three dilutions of a serum pool which had been standardised using enzyme immunoassay kits (Enzygnost-SP₃). These were gifted by Behringwerke AG but are no longer available. Within-assay coefficients of variation (CV) were determined by measuring each of these dilutions 80 times in a single assay. Between assay variation was determined by measuring each dilution in assays performed on several different days. The results are shown in Table I.

The sensitivity of the assay was 3 μ g/l (i.e. the absorbance of a sample containing 3 μ g α_2 -PAG/l was always greater than the mean absorbance value for incubation medium alone + 2 SD). The lowest concentration of α_2 -PAG in any of the samples tested was 8 μ g/l.

α_2 -PAG in serum and plasma

Venous blood samples were collected from 10 healthy donors and serum and

TABLE I

CV values obtained by testing dilutions of a standard serum in the assay system (see 'Assay Precision and Sensitivity')

Sample no.	(α_2 -PAG) in $\mu\text{g/l}^a$	CV within assay (%) $n = 80$	CV between assay (%)
1	10.5	6.2	11.6 ($n = 10$)
2	100	4.3	8.6 ($n = 40$)
3	250	5.9	11.8 ($n = 10$)

^a Concentration of diluted sera dispensed into the microclisa tray wells.

plasma were prepared from each sample. Alpha₂-PAG concentrations were measured in fresh samples and in samples which had been stored at -20°C . No significant differences were detected either between fresh and frozen samples or between serum and plasma. The protein is therefore unaffected by freezing and valid comparisons can be made between concentrations in sera (children's samples) and plasma (adults' samples).

α_2 -PAG in children

α_2 -PAG was measured in sera collected from 88 girls and 93 boys, grouped into a total of eight categories according to age and gender. A minimum of 20 samples was collected in each category. The results are shown in Fig. 2. No significant changes in α_2 -PAG concentrations occurred in either sex between the ages of 1 and 14 yr and there were no significant differences at any stage between boys and girls.

α_2 -PAG concentrations in a control group of otherwise normal epileptic children did not differ significantly from those in the larger study group which involved hospital inpatients.

α_2 -PAG in adults

α_2 -PAG was measured in plasma collected from 270 normal adults (129 females, 141 males) grouped into 12 categories according to age and gender. A minimum of 20 samples was collected in each category.

The results are shown in Fig. 3. α_2 -PAG concentrations increased with age in both sexes and were significantly higher in women in each age group.

α_2 -PAG in children and adults

Serum α_2 -PAG concentrations in children between the ages of 1 and 14 are also shown in Fig. 3 for comparison with the adult measurements. As stated previously, there was no significant difference between the sexes in this group. Beyond the age of 14, however, the differences between males and females in each age category were highly significant ($p < 0.001$). There was also a significant increase in α_2 -PAG concentrations in girls between the ages of 12-14 and 15-19 ($p < 0.05$). In boys, however, there was a significant decrease between these age categories ($p < 0.01$). The statistically significant differences between boys of 1 to 14 yr and adult males are shown in Fig. 4.

Discussion

The assay procedure described here is simple to perform and can be carried out conveniently in a working day if plates are coated overnight. In addition to having all the recognised advantages of an ELISA system (reviewed by Schuur and Van Weemen, 1977) this particular 'expanded' assay requires very low concentrations of reagents and is therefore inexpensive to carry out. The sensitivity obtained for the detection of α_2 -PAG (3 $\mu\text{g/l}$) is substantially greater than that achieved by other methods including radioimmunoassay (50 $\mu\text{g/l}$, [14]). The procedure is also extremely flexible, e.g. when results are required quickly we have successfully measured α_2 -PAG concentrations in 2 h by reducing incubation times to 30 min and using the peroxidase conjugate at a dilution of 1 : 5000. Greater sensitivity (at least as low as 1 $\mu\text{g/l}$) can also be achieved by increasing the conjugate concentration and, by this method, we have measured the very low levels of α_2 -PAG in sweat, urine, saliva, lachrymal secretions and seminal fluid. However, α_2 -PAG can be quantified in all serum samples using the standard procedure.

The trends in α_2 -PAG concentrations in adults observed here (Fig. 3) are very similar to those described by Folkersen [1] i.e. levels increase with age in both sexes and are consistently significantly higher in women.

When considering our study of α_2 -PAG in children's sera, certain reservations must be expressed, especially when comparing the results obtained with our normal adult data. For ethical reasons, we could not obtain blood samples from a large group of healthy children and we therefore measured sera drawn from in-patients of the Royal Aberdeen Children's Hospital. No attempt was made to exclude patients suffering from particular disorders since the results of studies measuring the effects of disease on α_2 -PAG concentrations are equivocal. For example, although certain authors report increases in α_2 -PAG in malignant disease [2,9,10], others find no change [15,16]. We believe that the trends described here do not differ markedly from those occurring in healthy children since the results obtained using a smaller group of otherwise normal epileptic children were very similar. Epileptic children receiving phenobarbitone treatment were excluded from the 'control' group since phenobarbitone has been shown to affect IgA levels [17] and α_2 -PAG is known to be synthesised by IgA-producing plasma cells [18].

As illustrated in Fig. 2, we found no significant difference in serum α_2 -PAG concentrations in boys and girls between the ages of 1-14 yr and no increase with age during this time. The first statistically significant increase occurs in girls between the age groups 12-14 and 15-19. This result would be expected on the basis of the known stimulatory effects of oestrogen on α_2 -PAG synthesis [2,3]. In males we found a significant decrease in α_2 -PAG levels between childhood and young adulthood (Fig. 4), suggesting that androgens may have an inhibitory effect on synthesis of this protein in man.

In this context it is interesting to note that testosterone appears to inhibit synthesis of the putative murine analogues of α_2 -PAG, namely PAMP-1 [19] and α_1 -PAP [20]. If this is also true in man, then the rise in α_2 -PAG concentrations which occurs in males over fifty years of age may be attributable to alleviation of

testosterone inhibition since the concentration of biologically active testosterone in the circulation starts the fall at this age, becoming minimal by the age of 70 [21].

It is interesting to speculate that the continued rise in α_2 -PAG levels in post-menopausal women first discussed by Damber et al [22] may also be attributable to this effect since androgenic steroids have been shown to fall to very low levels in women over fifty years of age [21].

It would appear therefore that α_2 -PAG synthesis may be stimulated by oestrogen and inhibited by androgens and clearly this merits further investigation.

Acknowledgements

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